THE EFFECTS OF GASTRIC AND HOMEOSTATIC AUTONOMIC AFFERENT REFLEXES ON CARDIAC AUTONOMIC EFFERENT ACTIVITY IN HEALTHY HUMAN VOLUNTEERS

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Abstract

The effects of gastric autonomic afferent reflexes on cardiovascular autonomic efferent activity are regarded as a direct neural effect of the activation of gastric receptors which send afferent inputs to the central nervous system (CNS) to cause various cardiovascular changes (Van Orshoven *et al.*, 2004; McHugh *et al.*, 2010; Girona *et al.*, 2014). However, the cardiovascular responses to liquid ingestion in humans may be related to gastric distension, volume loading effects, or to its osmotic proprieties. The purpose of this study was to investigate cross autonomic reflex function and to elucidate the effects of the resulting cardiac efferent autonomic activity in resting young healthy subjects. The ingestion of 300 mL of isothermic water increased both the cardiac vagal tone as indicated by increased RMSSD (mean 23.95 ± 20.50 msec increase, $p<0.05$) and sympathetic activity shown by increased QTc interval (mean 9.86 ± 8.59 msec increase, $p< 0.05$) during the first 40 minutes post-ingestion. These effects were absent with an identical volume of a physiological (0.9% w/v) saline solution which would increase plasma volume more, indicating that the cardiovascular responses to water drinking are influenced by its hypo-osmotic properties, rather than being related to the volume loading effects. Nevertheless, subjects responded to gastric distension with an ingestion of 300 mL of Fybogel solution with an increase in sympathetic activity during the first 20 minutes post-ingestion, but not in cardiac vagal tone. These results indicate that the mechanisms underlying the cardiovascular responses to water ingestion have additional components to the gastric stretch effect. Contrarily, the cold mediated sympathetic inhibition after drinking the same volume of

either cold water or cold Fybogel solution probably happened in the NTS where the two branches of the ANS meet for the first time during their central pathway (Kubin *et al.*, 2006; Thayer and Lane, 2009). In conclusion, the cardiovascular responses to water drinking are influenced by its hypo-osmolality properties and temperature, not by the volume loading effects.

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List Of Abbreviations

°C: Degree celsius

5- HT: 5-hydroxytryptamine (serotonin)

ACE: Angiotensin converting enzyme

ACh: Acetylcholine

AH: After-hyperpolarisation

ANS: Autonomic nervous system

ATP: Adenosine-triphosphate

AV: Atrioventricular

BP: Blood pressure

BPM: Beats per minute

C: Cervical

Ca^{2+}: Calcium ion

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

CGRP: Calcitonin gene-related peptide

Cl^-: Chloride ion

CMR1: Cold-and menthol-sensitive receptor1

CNS: Central nervous system

CO: Cardiac output

CRH: Corticotropin-releasing hormone

CVLM: Caudal ventrolateral medulla

CVS: Cardiovascular system

DBP Diastolic blood pressure

DH: Dorsal horn

DMN: Dorsal motor nucleus

DMNV: Dorsal motor nucleus of the vagus nerve

DRG: Dorsal root ganglion

ECF: Extracellular fluid

ECG: Electrocardiogram

EDTA: Ethylenediaminetetraacetic acid

ENS: Enteric nervous system

EPSP: Excitatory postsynaptic potential

FFT: Fast Fourier Transform

GABA: Gamma-amino butyric acid

GI: Gastrointestinal

GPCR: G protein couple receptor.

H^+: Hydrogen ion

HCl: Hydrochloric acid

HF: High frequency

HR: Heart rate

HRV: Heart Rate Variability

Hz: Hertz

ICH: International conference on harmonisation

iGluR: Inotropic glutamate receptor

IP_3: Inositol triphosphate

IPAN: intrinsic primary afferent neuron

J-receptor: Juxtapulmonary receptor

K^+: Potassium ion

L: Lumbar

LF: Low frequency

LPBN: Lateral parabrachial nucleus

M: Molar

MAP Mean arterial pressure

Mg^{2+}: Magnesium ion

mL: Millilitre

MMC: Migrating myoelectric complex

mmHg: Millimetre of mercury

msec: Millisecond

MV: Minute volume

NA: Nucleus ambiguous

$Na^{+:}$ Sodium ion

NG: Nodose ganglion

NMDA: N-methyl-D-aspartic

NO: Nitric oxide

NPY: Neuropeptide Y

NREM: Non-rapid eye movement

NTS: Nucleus tractus solitarius

nu: Normalised unit

OT: Oxytocin

OVLT: Organum vasculosum of the lamina terminalis

PACAP: Pituitary adenylate cyclase activating polypeptide

pH: Potential of Hydrogen

PIP_2: Phosphotidylinositol 4-5 biphosphate

PLC: Phospholipase C

PNN50: The number of pairs of successive NNs that differ by more than 50 ms

PNS: Peripheral nervous system

PO: Peppermint oil

PVN: Paraventricular nucleus

QTc: QT interval correction

RAA axis: Renin-angiotensin-aldosterone axis

RMSSD: Root mean square of the successive differences

RSA: Respiratory sinus arrhythmia

RVD: Regulatory volume decrease

RVLM: Rostral ventrolateral medulla

SA: Sino-atrial

SBP: Systolic blood pressure

SEM: Standard error of mean

SFO: Subfornical organ

SNS: Somatic nervous system

SON: Supraoptic nuleus

TPR: Total peripheral resistance

TRP: Transient receptor potential

TRPA: Transient receptor potential ankyrin

TRPC: Transient receptor potential canonical

TRPM: Transient receptor potential melastatin

TRPML: Transient receptor potential mucolipin

TRPN: Transient receptor potential NOMPC

TRPP: Transient receptor potential polycystin

TRPV: Transient receptor potential vanilloid

TSH: Tyroid stimulating hormone

TV: Tidal volume

UVLF: Ultra-very low frequency

VIP: Vasoactive intestinal polypeptide

VP: Vasopressin

VVR: Vasovagal reaction

α: Alpha

β: Beta

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CHAPTER 1. GENERAL INTRODUCTION

The Autonomic nervous system (ANS) is a branch of the nervous system that controls visceral functions of the body, including cardiac function, motility in the gastrointestinal (GI) tract, among many other vital activities, in order to maintain homeostasis within the body. Brainstem parasympathetic circuits that regulate the digestive functions of the stomach consist of sensory afferent vagal neurons, nucleus tractus solitarius (NTS), and the efferent neurons originating in the dorsal motor nucleus of the vagus (Travagli *et al*., 2006). Sympathetic control originates from cholinergic preganglionic nerve fibres in the intermediolateral column of the thoracic spinal cord, which impinge on postganglionic neurons in the celiac ganglion, of which the catecholaminergic neurons give the stomach most of its sympathetic supply. Therefore, the activation of gastric receptors conveys sensory information via afferent inputs to the NTS which in return sends efferent signals to the effectors such as heart and vasculature.

1. The Nervous System

The nervous system is a complex information-processing system which consists of the central nervous system (CNS) and the peripheral nervous system (PNS) and coordinates various activities of the body (Neary and Zimmermann, 2009). External and internal stimuli detected by sensory receptor cells, are transduced into electrical signals and conveyed via sensory afferent nerves to the CNS where the information is processed (Kumar *et al*., 2010). Neurons in the integrative centre are mostly interneurons which contact nearby neurons in the

brain, the spinal cord or the ganglia. The main integrative centres within the brain include the hypothalamus, which coordinates autonomic reflexes of the brainstem and spinal cord, and regulates homeostasis. The integrative centres also include the reticular formation which is a neural network of interconnected nuclei that are located throughout the brainstem and activates the system mediating consciousness and arousal via connections with specific nuclei of the thalamus, and the forebrain consisting of the cerebral cortex and subcortical structures (Oliveira-Maia *et al*., 2011). Processed information is then conveyed via either somatic or autonomic pathways of the efferent motor division to the effectors for an appropriate response (Nardone *et al*., 2013; Nomaksteinsky *et al*., 2013). The PNS is subdivided into somatic, autonomic, and enteric nervous systems (figure 1) and includes twelve pairs of cranial nerves, thirty-one pairs of spinal nerves, ganglia and sensory receptors. Somatic receptors convey sensory afferent inputs to the CNS where most of the impulses reach our awareness. In return, the CNS transmits via motor efferent division consciously controlled impulses, exclusively to the skeletal muscles, mediated by acetylcholine (ACh) to which the nicotinic skeletal muscle cell receptors are responsive and induce an opening of chemical-messenger-gated channels in the motor end plate for voluntary movements (Sine, 2012). The ANS consists of afferent neurons which convey sensory inputs to the CNS, and efferent neurons consisting of the sympathetic and parasympathetic branches conveyed to viscera effectors often without inducing our awareness (Barrett *et al*., 2010b). The ANS maintains homeostasis, adaptability, physiological flexibility, and assists the endocrine system in the modulation of reproduction within the body

via its reflexes (Tonhajzeroba *et al.*, 2013). However, the enteric nervous system (ENS) which is an agglomeration of neurons in the gastrointestinal tract (GI tract) capable of functioning independently from the CNS (Furness, 2000), communicates with the CNS (figure 1) (Cheng *et al.*, 2013). Two large categories of afferent neurons innervate the GI tract: Intrinsic neurons which do not have a direct connection with the CNS and extrinsic sensory nerves which transmit stimuli sensed within the GI tract to the CNS via either splanchnic or vagal neurons (Furness *et al.*, 2000). Vagal afferent neurons emerging from the nodose ganglion (NG), project to the medullary region of the brainstem, whereas splanchnic sensory neurons, arising from the dorsal root ganglion (DRG), project to the dorsal spinal cord (Holzer, 2001). These GI sensory neurons are sensitive to mechanical and chemical stimuli and express a large diversity of mechanical and chemical sensitive ion channels and the transient receptor potential (TRP) ion channels represent one such group that play a major role in various functions, including blood pressure (BP) and osmotic regulation (Holzer, 2009).

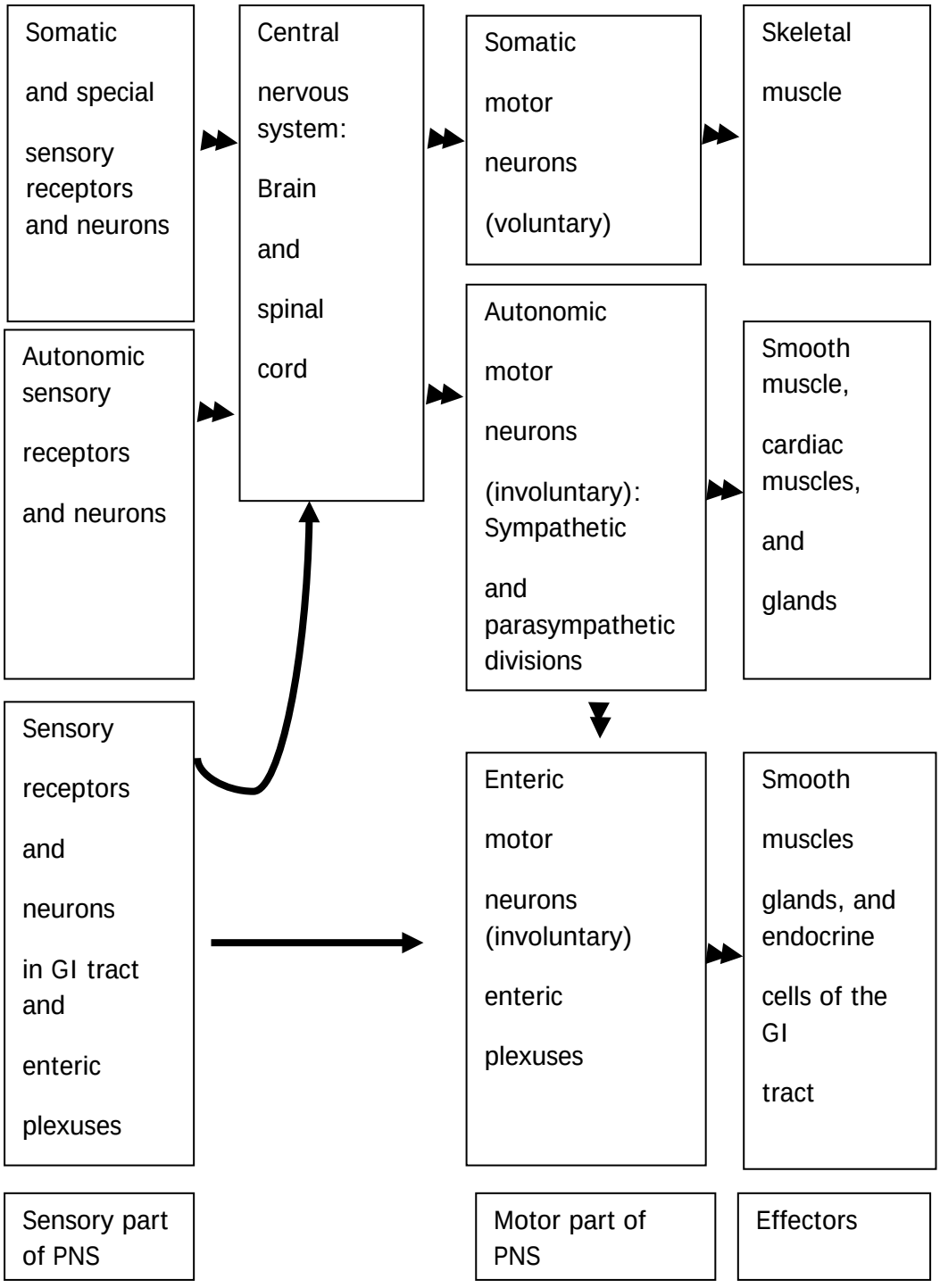

Figure 1: Organisation of the Nervous System. The CNS receives inputs from sensory receptors via sensory afferent neurons and sends output signals through motor efferent nerves (Furness, 2000).

1. 1. Autonomic Nervous System

From the time Galen (A.D. 130-200) described the morphology of the peripheral vegetative nervous system for the first time, to the time acetylcholine (Loewi, 1921) and noradrenaline (Euler, 1946) were discovered as chemical neurotransmitters involved in the vagal and sympathetic activities respectively, various research research has been performed to investigate the anatomy and the physiology of the ANS (Ackerknecht, 1974), including the study of Eustachius (1524-74) who regarded the vagus and sympathetic as two different nerves (Tissot, 1778). Contrarily, Galen considered what he called the sixth cranial nerve as the combination of what are called today the ninth (glossopharyngeal), tenth (vagus), eleventh (accessory) nerves and the sympathetic chain (Siegel, 1968). The autonomic afferent fibres convey impulses from sensory organs to the controlling centres in the CNS, including the medulla, the pons and the hypothalamus, while the autonomic motor pathways consist of two motor neurons in series (Pardo *et al.*, 2012). The first myelinated nerve fibres originating from the CNS extend their axons to either an autonomic ganglion or the adrenal medulla, whereas the second interconnecting with the first in the autonomic ganglion where it originates, extend the unmyelinated axons to the effectors (Westfall and Westfall, 2010). The sympathetic and parasympathetic branches of autonomic efferent nerves work in a reciprocal pattern or in a synergic fashion (Paton *et al.*, 2005) as will be explained later. The sympathetic nervous system (thoracolumbar division), consists of an output from neurons with cell bodies in the lateral horn of T1 to L2 or L3 segments of the spinal cord and is associated with fight-or-flight

responses (Westfall and Westfall, 2010). Visceral sympathetic afferent nerves are conveyed through the spinothalamic tract, the spinoreticular tract, and the dorsal column trajectory which may be considered as the primary pathways taken by ascending spinal visceral afferent neurons to the CNS (Saper, 2002). Most efferent preganglionic fibres synapse in the sympathetic ganglia along the paravertebral ganglia chains located either side of the vertebral column, whereas some fibres bypass the paravertebral ganglia to synapse in the prevertebral ganglia (the celiac ganglion, the superior mesenteric ganglion and the inferior mesenteric ganglion) (Westfall and Westfall, 2010). In addition, the remaining fibres travel via splanchnic nerve fibres to connect directly with the cells in the medulla of adrenal gland (Orban *et al*., 2015). Noradrenaline is the principal neurotransmitter at postganglionic endings, except the fibres innervating sweat glands which release acetylcholine (Waterhouse and Campbell, 2014). The ACh released by the preganglionic neurons activate nicotinic receptors in the ganglia, but the postganglionic neurons release either noradrenaline (or adrenaline), ACh, or dopamine to activate α and β receptors, muscarinic receptors, or dopamine receptors in the cardiovascular system (CVS), sweat glands, or renal vessels respectively (Cervi *et al*., 2014). Nevertheless, the parasympathetic nervous system (craniosacral division) decreases the activity of various organs (not all), including heart rate (HR) and BP (Beissner *et al*., 2013). Cranial parasympathetic sensory nerve fibres convey information to the brain via the oculomotor (III), facial (VII), glossopharyngeal (IX) and vagus (X) nerves, whereas pelvic viscera inputs are transmitted from the second to the fourth sacral spinal segments to the brain (Saper, 2002). The

preganglionic nerve fibres release ACh which stimulates nicotinic receptors, but within the wall of the effectors (such as heart and vessels), ACh activates muscarinic receptors (Saper, 2002). The visceral afferent information from the four cranial nerves terminates first in the Nucleus Tractus Solitarius (NTS), before being relayed by the parabrachial nucleus for ascending visceral inputs, which provides extensive projections to other sites of the brainstem, hypothalamus, forebrain, thalamus and cerebral cortex (Kaur *et al.*, 2013). In addition, the NTS and the parabrachial nucleus project to other targets that may give access to cortical area, including the direct projection from the gustatory parabrachial nucleus to the cerebral cortex which has an arousing influence on behaviour towards a food source (Saper, 2002). Most of the ascending spinal afferent neurons terminate either in lamina I or the deep layers of the dorsal horn (IV and V) or in the intermediate grey matter (layers VII and X) where some cells (layer X) may send ascending afferent neurons associated with visceral pain through the dorsal columns, which can reach the contralateral ventroposterior thalamic complex (Kaur *et al.*, 2013).

1. 2. Enteric Nervous System

The ENS contains almost as many neurons as the spinal cord (around 100 million) (Goyal and Hirano, 1996), and controls different functions in the GI tract, including motility, exocrine and endocrine secretions, microcirculation, and exchange of fluids across the mucosal surface (Surprenant, 1994). The ENS is organised into an interconnected network of neurons and glial cells that are grouped into ganglia located in two major plexuses; the myenteric (Auerbach's)

plexus and the submucosal (Meissner's) plexus (Furness, 2000). The myenteric plexus located between the longitudinal and circular layers, extends the full length of the GI tract, projects nerve fibres to the sympathetic ganglia (Kirchgessner and Gershon, 1990), and exerts control over digestive motility (Furness, 2000). Contrarily, the submucosal plexus, prominent only in the small and large intestines and situated in the submucosa (Furness, 2000), regulates the GI blood flow and controls epithelial cell function (Sasselli *et al.*, 2012). These functions are minimal in some regions of the GI tract such as the oesophagus where the submucosal plexus is sparse or even missing (Sasselli *et al.*, 2012), but receives an innervation from the vagal nerve (Furness, 2000). Neurons in the submucosal plexus innervate the muscularis mucosa, intestinal endocrine cells and submucosal blood vessels (Goyal and Hirano, 1996). The ENS neurons are subdivided into Dogiel type I neurons characterised by a single long axon and Dogiel type II neurons which have multipolar neurons (Goyal and Hirano, 1996). Their activities are modulated through the release of various neurotransmitters, including ACh, substance P, serotonin (5-HT), adenosine-triphosphate (ATP), gamma-amino butyric acid (GABA), vasoactive intestinal polypeptide (VIP), and nitric oxide (NO) (Sasselli *et al.*, 2012). The presence of reflexes in an isolated intestine after extrinsic neurons supplying the intestine have been cut (Furness *et al.*, 1995a) reveals the existence of intrinsic primary sensory afferent neurons (IPANs) in the intestine. The afferent neurons located in the myenteric and submucosal plexuses are less excitable due to the action of a long lasting after- hyperpolarizing potential that follows somal action potentials and act to dampen excitability and are called after-hyperpolarisation

(AH) neurons (Lomax *et al*., 2005). Myenteric AH neurons serve as intrinsic primary afferent nerve fibres of the ENS and generate prolonged or slow after-hyperpolarizing potentials. They are all type II neurons and use ACh as a primary neurotransmitter of the excitatory neurons, despite the presence of other neuromodulators which play a minor role, such as tachykinin, substance P, neuropeptides K and neuropeptides gamma (ɣ) (Lippi *et al*., 1998). Different sensory receptors have been identified in the mucosa including mechanoreceptors, thermoreceptors, osmoreceptors, and chemoreceptors (Furness, 2000). Mucosal chemoreceptors sensitive to chemicals (applied to the lumen), elicit bursts of action potentials in type II neurons in the myenteric plexus (Bertrand *et al*., 1997), whereas mechanoreceptors sensitive to mechanical stimuli, can be found in both submucosal and myenteric plexuses (Blackshaw *et al*., 2007). Smooth muscle cells activated through stretch-activated channels, induce intrinsic primary afferent neuron responses, and generate successive waves of peristaltic activity (Furness, 2000). The intrinsic sensory neurons with cell bodies within the NG convey signals to the CNS via vagal and splanchnic nerves (Goyal and Hirano, 1996). Information concerning the state of the GI tract such as pain and discomfort conveyed by afferent neurons, reaches consciousness, contrary to afferent signals associated with nutrient load which dot not reach consciousness (Furness, 2007). Nociceptive receptors responding to a high intensity of mechanical, thermal and chemical stimuli that can damage the tissue, are relayed by splanchnic primary afferent neurons with their cell bodies within the DRG, and use either the substance P or CGRP as neurotransmitters to convey inputs to the CNS (Goyal and Hirano,

1996). Splanchnic primary afferent neurons may exert direct action on the nearby GI effectors via long bifurcated axons, causing an axon-reflex (Goyal and Hirano, 1996). Unlike the classic reflex arcs consisting of a sensory receptor, afferent pathway, integration centre, efferent pathway, and an effector, the axon-reflex results from a stimulus applied to one branch of a neuron, which steps up an impulse that moves centrally to the point of division of the nerve, where it is reflected down the other branch to the effector organ, without reaching the integration centre (Yaprak, 2008). The vagus nerve conveys the parasympathetic motor fibres which control the secretomotor functions of the upper GI tract, whereas sacral nerves control function of the distal colon and the rectum (Goyal and Hirano, 1996). Therefore, the CNS exerts more direct control in the most proximal (oesophagus and stomach) and the most distal (recto sigmoid) parts of the GI tract and less direct control on the function of the small intestine and proximal colon. Medullary vagal preganglionic fibres innervating the gut have two parallel projections; an excitatory connection synapsing with cholinergic postganglionic nerves, and inhibitory, non-cholinergic neurons associated with non-cholinergic postganglionic neurons containing NO, responsible for the adaptive relaxation of the stomach mediated by vago-vagal reflex which is the outcome of gastric stretch in order to hold ingested food (Curro *et al.*, 2008). The adrenergic sympathetic fibres with their postganglionic cell bodies within the prevertebral ganglia (no adrenergic cell bodies in the enteric plexus), target the gastrointestinal sphincters, the submucosal blood vessels, the presynaptic cholinergic nerve endings and secretomotor neurons containing VIP (Wood,

1999) to control blood vessels (vasoconstriction), epithelial transport, motility, and enterochromaffin cells (Lundgren, 2000). In general, sympathetic activation induces the inhibition of gastrointestinal secretion, motor activity and contraction of the sphincters within the GI tract or blood vessels. The increase in sympathetic activity is capable of shunting blood from the splanchnic to the systemic circulation, known to suppress some digestive functions, including motility and secretion (Wood, 1999). Noradrenaline released from postganglionic neurons during sympathetic activation keeps the sphincters closed during the motility shut-down. Noradrenaline decreases the release of serotonin and substance P with presynaptic suppression of slow synaptic excitation as well as supressing the release of ACh at synapses in the enteric network (Wood, 2011). The activation of sympathetic neurons induces vasoconstriction associated with a decrease in blood flow in the crypts and muscles layers. The sympathetic control of epithelial transport and motility is also organised at a synaptic site because the sympathetic activation may inhibit local excitatory motor reflexes and/or extrinsic excitatory parasympathetic nervous activity. The stimulation of adrenergic receptors inhibits the release of the enterochromaffin contents, including serotonin, whereas the cholinergic influence attenuates the vasodilatation caused by mucosal mechanical stimulation (Lundgren, 2000). In addition, prevertebral sympathetic ganglia have neural connections which bypass the synaptic circuit within the intramural nervous system for rapid transfer of signals between different regions of the intestine (figure 2) because the signal within the ENS rarely travels more than

few centimetres without encountering a synapse that may slow the speed of transmission (Wood, 1999).

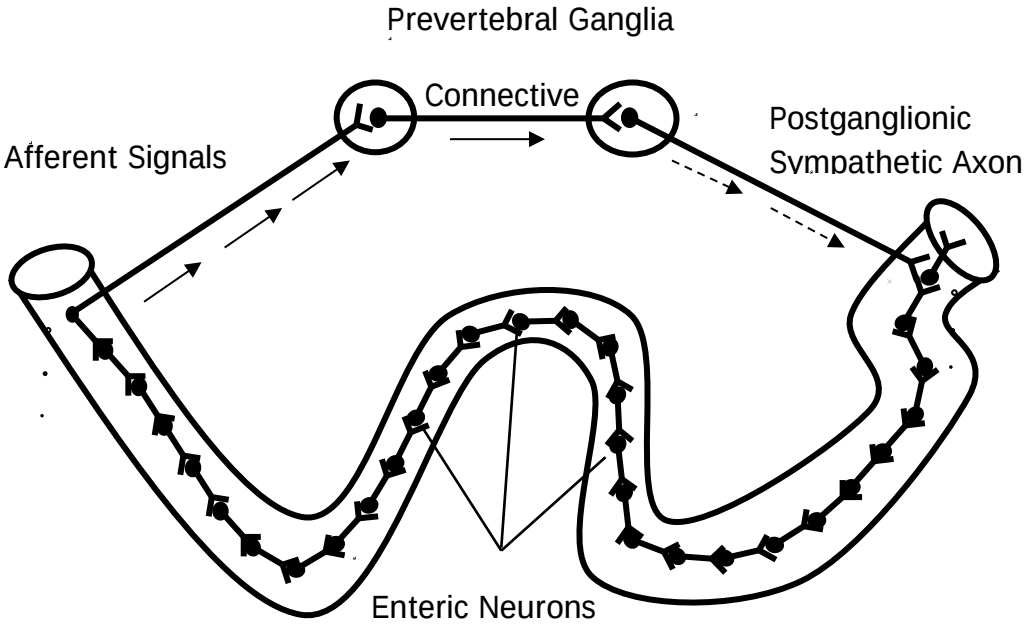

Figure 2: Rapid transfer of signals between separated regions of the intestine. The prevertebral sympathetic ganglia have neural connections for rapid transfer of signals between separated regions of the intestine. Afferent signals to the ganglia from one region of the intestine are relayed as inhibitory signals in postganglionic sympathetic fibres to another region (Wood, 1999). Dashed lines indicate inhibitory signals and solid lines show excitatory signals.

Therefore, intramural neurons synapse with postganglionic sympathetic neurons in the prevertebral ganglia, which in turn transmit signals to the bowel. The intrinsic neurons form plexuses with many connections within cardiac muscles (Amour, 2008), but the degree of influence of the intrinsic nerve plexuses on the efficacy of transmission at the parasympathetic ganglia and upon extrinsic autonomic postganglionic terminals remains obscure (Coote, 2013). Most motor intrinsic neurons exhibit tonic spike discharge (referred to as S neurons), lack of slow after- hyperpolarisation, and receive abundant fast

excitatory postsynaptic potentials (EPSPs). There are five different types of motor neurons; excitatory neurons of the gut muscles, inhibitory neurons of the gut muscles, secretomotor/vasodilator neurons, secretomotor/non-vasodilator neurons, and entero-endocrine cells neurons which include gastrin secreting endocrine neurons of the stomach (Furness, 2000). Three varieties of extrinsic motor neuron directly innervate effectors in the gut, including vagal motor neurons to the oesophagus, sympathetic neurons that innervate gut muscle, especially the sphincters, and noradrenergic vasoconstrictor neurons of arteries within the gut wall. There are other indirect motor extrinsic nerves, such as those that reach the gut through the vagus and pelvic nerves, and the sympathetic effects via myenteric and submucosal ganglia, via enteric intrinsic motor neurons (Furness, 2000). Enteric inhibitory neurons release different neurotransmitters, including NO, ATP, VIP, pituitary adenylate cyclase activating polypeptide-38 (PACAP), carbon monoxide, GABA, and neuropeptide Y (NPY) (Furness, 2000). The muscularis mucosa receives both excitatory and inhibitory neurons comparable in transmission properties to the neurons in the muscularis externa (Costa *et al.*, 1996). Motor sensory neurons have type I morphology, express excitatory activity through ACh and substance P, whereas the inhibitory action is mediated via VIP and NO neurotransmitters (Goyal and Hirano, 1996), and control gastrointestinal motility, secretion and probably absorption and act directly on a large number of effector cells, including smooth muscles, secretory cells and GI endocrine cells (Furness *et al.*, 2013).

However, the intrinsic interneurons located between afferent and motor neurons form multisynaptic pathways. There is one type of interneuron,

cholinergically mediated and formed by ascending neurons, involved in the propulsive reflexes in the gut (Furness, 2000). There are three types are descending interneurons having different chemical codings and use variety of neurotransmitters, including VIP, GABA, NPY, and 5-HT. The first type of neurons (ChAT/NOS/VIP neurons) are involved in local motility reflexes, the second type of nerve fibers (ChAT/SOM neurons) are implicated in conduction of migrating myoelectric complexes (MMCs) in the small intestine, and the third type of neurons (ChAT/5-HT neurons) are involved in secretomotor reflexes (Pompolo and Furness, 1998).

1. 3. Transient Receptor Potential Channels

The transient receptor potential (TRP) channels are members of the ion channel group of receptors activated by different mechanisms, such as receptor activation via G protein-coupled receptors (GPCRs), ligand activation by endogenous products of metabolism, and direct activation from changes in temperature, osmolality, mechanical and chemical stimuli (Ramsey *et al.*, 2006). These ion channels are found in most mammalian tissues, including the plasma membrane of the GI tract, liver, portal system, adipocytes, sensory nerves, and the osmosensitive regions of the CNS including hypothalamus (Ahern, 2013). The 28 mammalian members of the super-family TRP channels structurally similar to voltage-gated K^+ channels are morphologically grouped into seven subfamilies: Canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), polycystin (TRPP), mucolipin (TRPML), and ankyrin (TRPA) (Clapham, 2003), with the seventh family; NOMPC (TRPN) found only in invertebrate and fish (Nilius and Owsianik, 2011). Each channel is a tetrameric complex consisting of

identical or similar monomeric domains, and each domain has six transmembrane segments (S1–S6), cytosolic amino (N), carboxyl (C) termini, and a pore region located between the S5 and S6 transmembrane domains (Ahern, 2013). They operate as non-selective cation channels, freely conducting Na^+ and K^+ ions and give access to varying degrees Ca^{2+} and Mg^{2+} ions (Cenac *et al.*, 2008). Only a few are highly Ca^{2+} selective and a few more are permeable to highly hydrated Mg^{2+} ions (Nilius and Owsianik, 2011). Activated TRP channels induce depolarisation of the cellular membrane, which in return activates voltage-dependent ion channels, resulting in a modification of intracellular Ca^{2+} concentration (Brierley *et al.*, 2008). The Phospholipase C (PLC) signalling and lipids regulate TRPC and TRPV channels; TRPV 1- 4, TRPM 2, 4, 5, and 8, and TRPA 1 are sensitive to temperature, whereas TRPV4 channel is osmotically sensitive (McHugh *et al.*, 2010; Ahern, 2013). TRP ion channels activated by temperature, osmolality and the mechanically activated TRP channels are of interest in this study as they are associated with various autonomic efferent activities investigated in our different experiments (table 1). Other TRP channels, including TRPC receptors (associated with anorexigenic signals), TRPM5 receptors (expressed in taste receptor cells) (Ahern, 2013) are not explicitly included here as they will not be exploited in our study.

Channel subunit	Characteristics
TRPV1 receptors	Capsaicin sensitive nociceptors expressed mostly in sensory neurons. Can also be activated by a wide range of stimuli, including temperature, changes in pH, osmotic and mechanical impulses (Green, 2005; Dhaka *et al.*, 2009).
TRPV4 receptors	Osmotic sensitive ion channels (McHugh *et al.*, 2010) which can also be mechanical and temperature-gated channels (Becker *et al.*, 2005). TRPV4 receptors are expressed in different locations within the body, including the GI tract, mensenteric vessels, liver (Liedtke and Kim, 2005).
TRPM8 receptors	Innocuous cold sensitive receptors expressed in primary sensory neurons, with some nociceptive properties (Tsuzuki *et al.*, 2004). TRPM8 receptors are found in various locations, including the DRG, the GI tract, and vasculature. These receptors can also be activated by eucalyptol, menthol, and icilin (Ahern, 2013).
TRPA1 receptors	Noxious cold-thermo-sensitive receptor and can generate a cold-induced contraction in colon (Nilius and Owsianik, 2011).

TRPP	TRPP receptors form complexes that act as mechanosensory transducers in a variety of biological functions (Delmas, 2004)

Table 1: Various TRP ion channels activated by different stimuli during in our different experiments. TRPP, TRPV4, TRPA1, and TRPV1 are reported to be activated by mechanical impulses (Huang, 2004; Nilius and Owsianik, 2004).

The stimulation of TRPV1 sensory receptors is associated with maintenance of mucosal integrity and in colitis-induced gastroparesis, it has been reported a TRPV1-mediated visceral afferent nerve sensitization as an alternative for visceral hyperalgesia, explaining its use in the treatment of GI disorders in traditional Chinese medicine. Water extracted from the Evodia rutaecarpa plant (used in Chinese medicine) inhibits the intestinal transit and has protective effects on acetylsalicylic acid, stress and ethanol-induced gastric mucosa injury by activating TRPV1 receptor and the release of endogenous CGRP (Liao *et al.*, 2011). In the cardiovascular system, TRPV1 receptor activation plays a role in BP control and in protection against cardiac injury via the release of substance P and nitric oxide, mediating the vasodilatation of coronary arteries (Deng and Li, 2005). TRPV4 receptors play a role in regulatory volume decrease (RVD) in response to hypotonicity, in order to maintain the osmotic homeostasis at the cellular level, which enables the cell to regain its former volume although the cell remains in a hypotonic environment by loss of ions, mainly K^+ and Cl^-, followed by loss of water giving a mechanism of volume reduction (Becker *et al.*, 2005). The activation of TRPV4 induces the release of NO, CGRP and

substance P, potent vasoactive compounds which work as neurotransmitters (Grant *et al.*, 2007). However, TRPM8 receptors originally cloned from the prostate (Tsavaler *et al.*, 2001), can be activated by cold temperature (<25°C) at peripheral nerve endings in a subset of thermosensitive Aδ- and C-fibre neurons (Campero *et al.*, 2001). TRPM8 knockout (TRPM8 $^{-/-}$) mice are insensitive to ambient cooling (Ahern, 2013), indicating TRPM8 ion channel to be responsive in sensing cold temperatures in the cold range (<25°C). The activation of TRPM8 receptors in the tissues that are not exposed to any temperature variations, such as prostate, raised a possibility that TRPM8 receptors may be stimulated by some endogenous ligands (Abeele *et al.*, 2006). Phosphatidylinositol 4-5, bisphosphate (PIP_2) is reported to be such a factor since it is capable of restoring menthol-activated TRPM8 current after its depletion in excised patches (Abeele *et al.*, 2006). The stimulation of TRPM8 receptors by PIP_2 maintains the response to cold or menthol and high concentration of PIP_2 can allow TRPM8 receptor channels to open even at temperature 32-37 °C (Rohacs *et al.*, 2005). Although TRPM8 receptors are expressed in visceral organs and use vagal afferent neurons innervating the GI tract to send temperature-related information to the NTS (Zhang *et al.*, 2004), its physiological role is more important in the skin and mucosal surface (Harrington *et al.*, 2011) where the movement of the calcium ions across the sensory nerve-cell membrane regulates the membrane potential and the electrical activity of these cold receptors (Rebocho *et al.*, 2014). In the skin or mucosal surface, cold receptors show oscillations in the membrane potential caused by influx and efflux of calcium ions and whenever the membrane

potential depolarises, a bursting discharge of action potential fires (Eccles *et al.*, 1994). Animal experiments demonstrated that a decrease in external calcium concentrations by administration of a calcium-chelating agent EDTA slows the efflux of calcium from cold receptors of nasal skin, because the influx of calcium activates the efflux mechanism (Schafer *et al.*, 1982). Contrarily, an intravenous administration of calcium solution speeds the efflux of calcium from cold receptors, and induces an increase in the frequency of warm-receptor discharge and a depression in the discharge of cold receptors in the nasal area of a cat (Eccles, 1994). The efflux of calcium from cold receptors induces hyperpolarisation and inhibits the discharge of action potential (Hensel and Schafer, 1974). Binding to TRPM8 receptors, the cold response activating agent menthol exhibits calcium-channel blocking properties and interferes with the movement of calcium across the cell membrane by inhibiting the efflux of calcium from these cold receptors and increases electrical discharge from cold receptors in the same way as EDTA (Hawthorn *et al.*, 1988). The inhibition of potential-dependent calcium currents by menthol indicates that menthol reduces the influx of extracellular calcium ions in smooth muscle (Grigoleit and Grigoleit, 2004). Menthol blocks currents through the low-voltage-activated Ca^{2+} channel, and facilitates inactivation gating of the classical high voltage-activated Ca^{2+} channel (Grigoleit and Grigoleit, 2004). The antispasmodic properties of PO come from the inhibitory action of menthol on gut smooth-muscle calcium conductance which decreases the influx of extracellular calcium through potential-dependent channels, while having no effects on intracellular mobilisation of calcium (Hawthorn *et al.*, 1988). The use of PO in irritable bowel

syndrome may be due to the relaxing effect of peppermint on the intestinal smooth muscle obtained by the interference of menthol with the movement of calcium across the cell membrane (Grigoleit and Grigoleit, 2005). However, behavioural studies have demonstrated that menthol has neuronal actions independent of its effects at peripheral nerve endings (Tsuzuki *et al.*, 2004) as described in chapter 5.

1. 4. Cardiovascular System and Autonomic Control

The cardiac sympathetic supply, although receiving projections from different areas of the CNS, including the insula, hypothalamus, and amygdala, originates from the stellate ganglia, and innervates the four chambers of the heart, nodal and conducting tissues (Brack *et al.*, 2009). The old conception indicating the cardiac sympathetic efferent neurons conveyed to heart via NG in the nerve bundles (Heibecker and O'Leary, 1933a) is abandoned because the cervical sympathetic nerve that passes through the NG supplies the bronchial circulation and pulmonary blood vessels (Daly and Evans, 1953), not the heart (Coote, 2013). The idea that sympathetic neurons innervate ventricular muscles, atrial, sino-atrial (SA) node, atrioventricular (AV) node and conducting tissues, whereas parasympathetic nerves densely innervate the atria and nodal tissues, with some sparse fibres on the ventricles (Brodde *et al.*, 2001) is not correct. Several observations have indicated the presence of cardiac vagus neurons supplying the ventricles and having influence on ventricular rate and rhythm, and contractility (Ulphani *et al.*, 2010). The investigation of vagus neurons on the heart using the thiocholine method used to identify the presence of acetyl-cholinesterase, the enzyme responsible for hydrolysing acetylcholine, showed

the presence of cardiac cholinergic innervation widespread all over the four chambers of the heart (Rysevaite *et al*., 2011). The thiocholine ester used as substrate is hydroiysed by cholinesterase, and captured by Cu^{2+} ions, precipitating as colourless cooper thiocholine, which is converted to brownish by treatment with yellow ammonium sulphite (Karnovsky and Roots, 1964). The epicardial and endocardial surfaces of the atria and ventricles are richly innervated by both parasympathetic and the sympathetic nerves (Ulphani *et al*., 2010). Cardiac parasympathetic neurons reduce cardiac functions via decreased excitability at nodal tissues and reduction in force of atrial contraction and in rate of atrioventricular conduction (Vaseghi and Shivkumar, 2009). The activation of vagal neurons to the heart is capable of decreasing both HR and atrio-ventricular conduction independent of sympathetic activity via NO release at presynaptic ending (Conlon *et al*., 1996). Cardiac vagus nerves on the ventricles have a protective effect on the vulnerability to ventricular fibrillation, reported to be attenuated by atropine, and prevented by adrenergic blocking of the sympathetic nervous system in animal models (Verrier and Lown, 1984). Parasympathetic action decreases the action potential duration and effective refractory period of atrial muscle cells, reducing the threshold for fibrillation (Liu and Nattel, 1997). In the absence of sympathetic tone, vagal stimulation causes the lengthening of the effective refractory period recorded from the ventricle and depresses the force of ventricular contraction (Brack *et al*., 2011). This mechanism is preeminent where the sympathetic tone is high, due to the accentuated antagonism effect involving cholinergic-induced formation of NO (Paterson, 2001) as will be explained later. The ventricular parasympathetic

action is regulated in two different ways. The first is a cholinergic-muscarinic action on the contraction rate and ventricle effective refractory period and the second is an independent effect of NO release independently of ACh action at M_2 post-junctional receptors (Brack *et al*. 2009). The NO released from cholinergic fibres acts at a different post-junctional site to ACh (Brack *et al*., 2011). Therefore, the vagally mediated anti-arrhythmic effect must be used by a selected population of parasympathetic postganglionic nitrogenic nerves (Hoover *et al*., 2009). This nitrogenic vagal pathway, different from the classic cholinergic one, has been established in the parasympathetic control of GI tract (Brack *et al*., 2011). Atrial and ventricular effects of vagal neurons are related to the excitation of ganglion cells found in discrete clusters on both the atria and its epicardium, and in the ventricular septum, targeting different regions of the heart within the four chambers. The autonomic imbalance may be associated with an over activity of either sympathetic or parasympathetic nervous systems (Samuels, 2007). Many reflex pathways such as the baroreflex are mediated by the ANS so that surgical denervation or pharmacological blockade of the vagal drive to the heart can elicit an increase in HR, indicating vagal influences being dominant over sympathetic influences at rest (Thayer and Lane, 2007).

1. 4. 1. Baroreflex Control on Cardiovascular System

The movement from a supine to an upright position requires adjustments in blood flow and BP, and these adjustments are coordinated by sympathetic nerves in conjunction with parasympathetic modulation of heart rate (Charkoudian and Rabbitts, 2009). Without such autonomic regulation, blood flow to the brain would fall below autoregulatory limits, and standing up would consistently cause syncope. The arterial baroreflex is a feedback mechanism working to buffer acute variations of BP by regulating the cardiac output and the total peripheral resistance, known to determine the arterial BP (Benarroch, 2008). The baroreceptors mainly located in the aortic arch and internal carotid sinus (at the bifurcation of external and internal carotids), are sensitive to both the absolute pressure and the rate of increase in BP within the vessels and mediate the regulation of BP and HR (Kregel *et al.*, 1990). The carotid sinus baroreceptors are innervated by the glossopharyngeal nerve (IX cranial nerve) which synapses in the NTS, whereas the aortic arch baroreceptors innervated by the aortic nerve which combined with the vagus nerve (X cranial nerve), also travel to the NTS (Davos *et al.*, 2002). The NTS modulates the activity of sympathetic and parasympathetic neurons in the medulla, which regulate the autonomic control of the heart and blood vessels. With an increase in BP, baroreceptors sense stretch in the sino-aortic structures and send impulses to the NTS in the medulla via the X and IX neurons (Benarroch, 2008). The barosensitive neurons within the NTS initiate a parasympathetic pathway by projecting to vagal parasympathetic neurons in the nucleus ambiguous (NA) (Kirchheim, 1976). Increased activation of these neurons elicits bradycardia by

decreasing the sinoatrial node pacemaker cells discharge rate (Benarroch, 2008). In parallel, the NTS communicates excitatory glutamatergic inputs to the caudal ventrolateral medulla (CVLM), which in return activates GABAergic interneurons that provide rapid inhibition of synaptic premotor neurons within the rostral ventrolateral medulla (RVLM) (Schreihofer and Guyenet, 2003). The RVLM contains a collection of neurons that innervate preganglionic sympathetic neurons of the spinal cord for sympathetic reflexes (Guyenet, 2010) elicited by cardiopulmonary receptors and descending inputs from the hypothalamus and higher in the neuraxis (Guyenet, 2006). These presympathetic neurons of the RVLM activate the intermediolateral nucleus of the spinal cord which activates the sympathetic output (figure 3). During baroreflex action, the RVLM is inhibited by GABAergic neurons from the CVLM which therefore inhibits sympathetic cardiovascular activity. The excitatory glutamatergic effect of the RVLM causing sympathetic activation is continuously restrained by the inhibitory input from the CVLM, mediated by the inhibitory neurotransmitter GABA. The Area postrema that is blood-brain barrier deficient is accessible by angiotensin II that increases its sympathetic activity via RVLM (Carlson and Wyss, 2008). The RVLM appears to have an especially dense distribution of neuronal angiotensin II receptors and when activated by angiotensin II during a reduced BP, induce an increase in sympathetic activity (Zucker and Gao, 2005). Other nuclei involved in cardiovascular regulation, including PVN, parabrachial nucleus, and the NTS have the existence of an endogenous brain renin angiotensin signalling system supported by the widespread distribution of neuronal angiotensin II receptors (Carlson and Wyss, 2008). In addition, the

trigeminal islands also play a role in central vagal afferent signalling (Berthoud *et al.*, 2000). The dorsal motor nucleus of the vagus nerve (DMNV), the area postrema and the medial subnuclei of NTS form the alimentary canal, whereas the best viscerotropic segregation is between cardiac and pulmonary afferents lateral subnulei of NTS (Pamidimukkala *et al.*, 2002). The neurotransmitters used by primary vagal afferent fibres also include the CGRP and substance P residing in jugular ganglion. Contrarily, there are a few sensory neurons in the NG containing neuro peptides (Berthoud *et al.*, 2000). Furthermore, there is strong evidence for L-glutamate acting through both N-methyl-D-aspartic (NMDA) and non-NMDA ionotropic glutamate receptors (iGluRs) in cardiovascular vagal afferents (Berthoud *et al.*, 2000).The preganglionic cardiac neurons mainly arise from two nuclei in the caudal medulla oblongata (Panneton *et al.*, 2014). The first preganglionic cardiac small myelinated (B fibres) fast neurons located in the posterior ventrolateral NA form about 80% of cardiac vagal neurons and can powerfully reduce HR, conduction and power of contraction. The second group of cardiac vagal neurons (20%, slow C fibres) originating from the DMNV and a scattering of neurons in an intermediate zone, have slowly conducting unmyelinated axons (Garcia-Perez and Jordan, 2001). The DMNV fibres have a more irregular non-respiratory-dependent discharge and are reported not be affected by baroreceptor and chemoreceptor signals (Jones *et al.*, 1998), contrary to the NA cardiac neurons which display a respiratory rhythm and receive baroreceptor and chemoreceptor inputs (Griffioen *et al.*, 2007), indicating that different cardiac functions may be mediated by different phenotypes of cardiac vagal preganglionic neurons. The

activation of two different plexuses within the heart; one at the junction of the superior vena cava and right atrium and the other at the intercession of the pulmonary veins and left atrium, induces either a bradycardia, or reduced atrio-ventricular transmission respectively (Sampaio *et al*., 2003). These two different phenotypes of cardiac vagal preganglionic neurons in the medulla are either inhibited by lung inflation (NA cardiac neurons) or not (DMNV cardiac nerve fibres) in connection with the type of cardiac vagal neuron that is activated (Sampaio *et al*., 2003). Thus, the baroreflex induced by increased BP simultaneously provokes an indirect decrease in sympathetic tone and a direct increase of parasympathetic nervous activity to return HR, BP and atrioventricular conduction to normal. Afferent baroreceptor input can also be transmitted to the thalamus via the interconnections between the NTS and the reticular formation (Rau and Elbert, 2001). The NTS directly projects to both the limbic structures (hypothalamus and the amygdala) and the lateral parabrachial nucleus (LPBN) (Schreihofer *et al*., 2005). The LPBN also projects to the hypothalamus and the amygdala, allowing the baroreflex input to reach the limbic system via indirect route (Benarroch, 2008). Furthermore, the LPBN extends some fibres to the lateral ventroposterior thalamus, allowing baroreceptor inputs to reach the cortex via the thalamus (Schreihofer *et al*., 2005). Results from some observations have also demonstrated the presence of both the prefrontal and the somatosensory cortices integrating baroreceptor input (Wong *et al*., 2007).

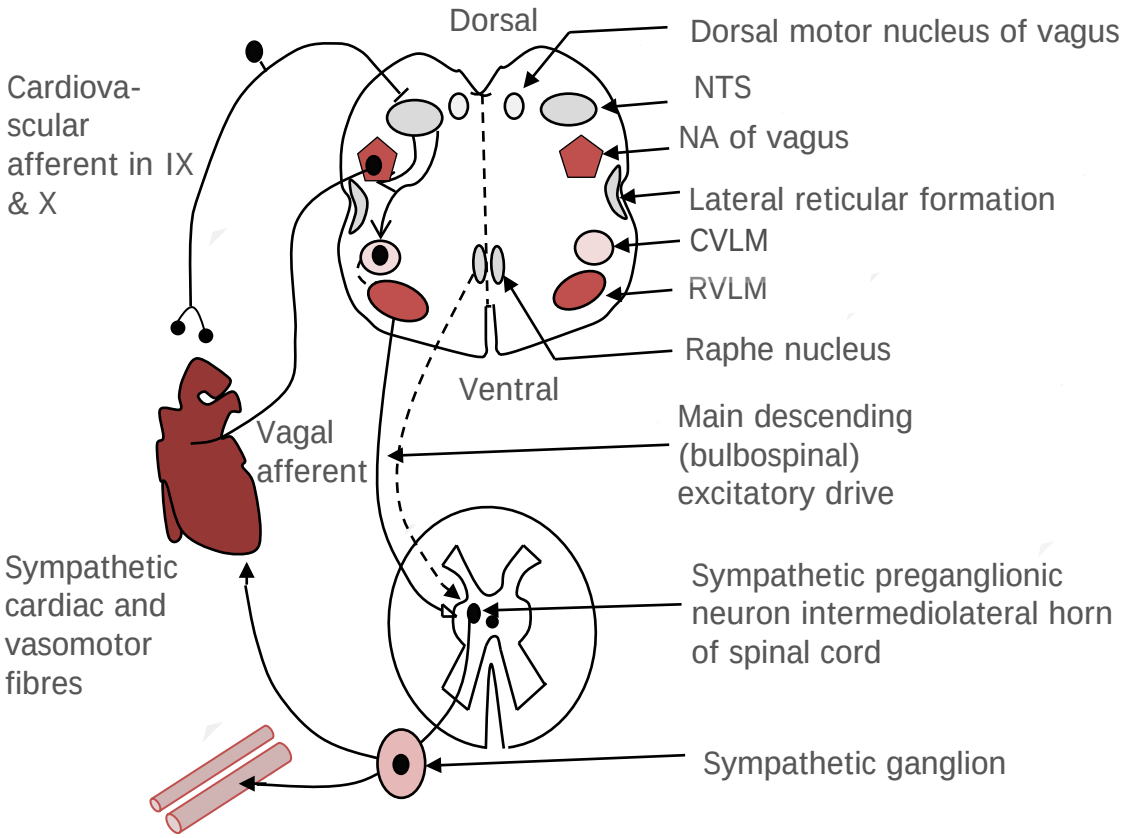

Figure 3: Baroreflex control of the HR and BP. The NTS activates the Nucleus Ambiguous to increase parasympathetic tone and inhibits the sympathetic activation by the RVLM via the activation of CVLM (Schreihofer and Guyenet, 2003). There is a more direct descending inhibitory influence on the spinal sympathetic nerve fibres coming from the raphe nuclei. Dashed lines indicate inhibitory pathways.

1. 4. 2. Other Reflexes on the Cardiovascular Control

Vagal dominance over sympathetic tone on the heart at rest can be ascribed partly to the tendency for vagally released ACh to inhibit sympathetic activity by inhibiting postsynaptic processes and/or through a presynaptic suppression of the release of noradrenaline from synaptic nerve terminals (Sun, 1995). The reciprocal control of cardiac vagal and sympathetic nervous activity described

during baroreflex response (Koizumi *et al*., 1985), is now considered as an exception rather than the rule (Paton *et al*., 2006). Many other reflexes involving simultaneous co-activation of both autonomic limbs of the ANS (Yang and Levy, 1992), include reflex responses evoked by stretching of the sinus node region of the right atrium, peripheral chemoreceptor reflex, diving reflex, oculocardiac and somatic nociceptor reflex responses (Koizumi and Kollai, 1981). There is now more evidence about reciprocal, independent or nonreciprocal (co-activation or co-inhibition) activities of the two branches of the ANS (Paton *et al*., 2005). An increased HR could arise from a decreased vagal tone, an increased sympathetic efferent activity or sympathetically dominant co-activation of both branches of ANS (Bernston *et al*., 1993). These complex interactions can be mediated either centrally or peripherally (Paton *et al*., 2005).

1. 4. 2. 1. Peripheral Control

In general, the sympathetic and parasympathetic divisions of ANS exhibit antagonist effects on various aspects of the performance of the heart (White and Raven, 2014). However, the HR activity may be influenced by complex interactions between the parasympathetic and sympathetic activities in animals, including accentuated antagonism and reciprocal excitation (Levy, 1971) During the accentuated antagonism, the greater vagally mediated decreased HR and heart contractility in the presence of sympathetic activation (Levy and Zieske, 1969) is due to the exaggerated effect of ACh in the presence of noradrenaline, inducing a predominance of vagally mediated cardio-inhibitory action over adrenergically mediated cardio stimulation (Hollenberg *et al*., 1965). ACh

inhibits the cardio- stimulatory effects of sympathetic stimulation either by a cholinergically mediated decrease in the amount of noradrenaline released from sympathetic terminals or by a cholinergic attenuation of the magnitude of the response to adrenergic activation by inhibiting the synthesis of postsynaptic cAMP (Burn and Rand, 1965; Brack *et al*., 2006). Noradrenaline release during sympathetic activation accelerates the synthesis of intracellular cAMP, which is responsible for atrial and ventricle inotropic activities (Laraia and Sonnenblick, 1971; Epstein *et al*., 1971). This mechanism is probably inhibited by the ACh released during vagal activity that elicits a proportionately larger decrease in cAMP content in the atrial tissue but a lesser reduction in the ventricular tissue (Levy, 1971). The depolarisation at the parasympathetic nerve endings induces the formation of NO which facilitates vagal release of ACh and inhibits sympathetic activity via the cholinergic intracellular elevation of cGMP that inhibits cAMP and leads to reduction of the sympathetic adrenergic-activated hyperpolarisation activated slow depolarizing (I_f current) and L-type Ca^{2+} current in the myocyte (Levy and Zieske, 1969). ACh exerts a muscarinic inhibition of noradrenaline release from sympathetic nerve fibres to the heart at higher concentrations which can be blocked by atropine (Levy, 1971), whereas at lower concentrations, ACh induces the release of noradrenaline from sympathetic nerve terminals in animal models (Burn, 1967).

Experiments carried out in dogs have demonstrated that one minute of sympathetic stimulation alone increased HR by 90 ± 7 beats/minute (mean ± SE), whereas one minute of vagal stimulation reduced HR by 67 ± 5 beats/ minute (mean ± SE), giving an algebraic sum of an increase of 23 ± 2 beats/

minute (mean ± SE) (Yang and Levy, 1992). However, a concomitant para-and-sympathetic activation for one minute reduced HR by 35 beats/ minute (Yang and Levy, 1992). These degrees of HR changes could be influenced reflexly or by the action of higher centres (Yang and Levy, 1992). This nonlinear summation known as accentuated antagonism (Levy, 1971) demonstrates a vagally mediated decrease in HR despite the simultaneous sympathetic stimulation. The opposing effect of sympathetic and parasympathetic activation does not obey to the algebraic summation because many non-adrenergic and non-cholinergic substances, including neuropeptides are released with noradrenaline and ACh (Bartfai *et al.*, 1988). The neurotransmitters released from nerve fibres of one autonomic division might influence the release of transmitters from the nerve endings of the other division (Muscholl, 1980), because the postganglionic sympathetic and vagal nerves fibres lie often side by side in the wall of the heart (Potter, 1985). Accentuated antagonism can be observed in the peripheral chemoreceptor reflex response, diving response, and oculocardiac reflex response (Angell-James *et al.*, 1969; Boscan and Paton, 2002). This vagally induced chronotropism easily overcomes sympathetic influences that preferentially target inotropic mechanisms despite their coincident activation (Koizumi and Kollai, 1981). The functional significance of this cardiac sympathetic and parasympathetic co-activation results in a greater increase in cardiac output due to the vagally mediated decrease in HR that allows an efficient ventricular filling and a stronger sympathetically mediated increase in the myocardium contractility (Paton *et al.*, 2005). During the diving test, the vagally mediated decrease in HR (approximately 20 beats/ minute

reduction) (Caspers *et al.*, 2011; Foster and Sheel, 2005) is an adaptive reaction that protects the heart from hypoxia subsequent to insufficient oxygen availability because of the inability of the heart to respire anaerobically on the one hand and its inherently high metabolic rate on the other (Paton *et al.*, 2005). During the decrease in HR in the diving the reflex, the non-neural mechanisms (Bowditch effect) causing a substantial decrease in ventricular contractility (Zurbuchen *et al.*, 2014) are compensated by a sympathetically mediated increase in myocardium contractility to optimize the stroke volume, similar to the chemoreflex situation (Paton *et al.*, 2005). The delivery of oxygen to the heart may be regulated by a fall in coronary vascular resistance which allow blood outflow into coronary arterioles (Paton *et al.*, 2005). Contrarily, when sympathetic activation is initiated first, the parasympathetic predominance to combined stimulation decreases progressively with the increase of antecedent sympathetic activation (Warner and Levy, 1990) because of the inhibition of ACh by NPY released from the sympathetic nerve endings, combined with noradrenaline during the sympathetic activation, and to the exogenous NYP (Potter, 1987; Warner and Levy, 1989). Therefore, the greater inhibition of vagal transmission is decreased by the longer antecedent sympathetic stimulation by cumulative amount of NPY released from the sympathetic terminals into the cardiac interstitium (Yang and Levy, 1992). A period of intense sympathetic activity would be the key to stop or decrease the vagal predominance on sympathetic activity after the cessation of the antecedent sympathetic action (Matthew and Yang, 1992), because the concurrent vagal activity must have inhibited the release of NPY from the

sympathetic nerve that could affect the cardiac response to the vagal action. Conversely, intense vagal stimulation alone potentiates the chronotropic response to subsequent vagal test stimulations, probably due to the increased amount of ACh released by the vagus nerve (Revington and McCloskey, 1990). However, during the reciprocal excitation, cholinergic activation has been reported capable of releasing noradrenaline from chromaffin or sympathetic ganglion cells located within heart muscles in animal models (Hoffmann *et al.*, 1945). Therefore, cardiac vagal efferent activation might supply synergetic co-activation to sympathetically mediated chronotropic influences, leading to a paradoxical vagally mediated tachycardia (Levy *et al.*, 1969). This initial transient vagal activation that returns to baseline level before the sympathetically mediated tachycardia has fully developed, is not involved in the maintenance of the tachycardia (Paton *et al.*, 2005). The tachycardia maintenance may be related to the release of neuropeptides from post-ganglionic vagal endings, including VIP, NPY, phenyl-histidine-isoleucine, substance P, and enkephalin co-released with ACh (Lunberg, 1979) or to the neuropeptides released from intrinsic cells to the cardiac ganglia (Paton *et al.*, 2006). Some of them, including VIP and phenyl-histidine-isoleucine have the same effects as noradrenaline in increasing HR and can modulate the efficiency of noradrenaline release from cardiac sympathetic terminals (Lunberg, 1976). Furthermore, the stimulation of sympathetic nerves to the heart might release the ACh from parasympathetic nerve fibres (Hashimoto *et al.*, 1970). Contrarily to the baroreflex that is more regulatory and homeostatic, other reflexes appear to be more protective by stimulating both autonomic outflows to the heart

concomitantly. The initial vagally mediated bradycardia and the subsequent tachycardia (partially attributed to vagal withdrawal) during the startle reflex could be evoked by sympathetic and parasympathetic co-activation (Abdeen *et al.*, 1995).

1. 4. 2. 2. Supramedullary Control of the CVS

Cardiopulmonary vagal and sympathetic afferent neurons interact firstly in the NTS during their central pathways so that sympathetic afferents are impeded when cardiac vagal afferents were stimulated simultaneously (Tjen-A-Looi *et al.*, 1997). However, hypothalamic interactions have been described during some reflexes, such as centrally evoked defence responses that elicit different cardiac responses depending on the stimulated site of the hypothalamus. Different responses may be observed, including reciprocal (sympathetic activation /vagal inhibition or vice versa) and non-reciprocal (co-activation or co-inhibition) effects (Koizumi and Kollai, 1981). For instance, stimulation of the defence and alerting areas of the hypothalamus produces a large increase in sympathetic activity and simultaneous inhibition of vagal tone, whereas stimulation of the anterior hypothalamus induces an enhancement of vagal tone and inhibition of sympathetic efferent activity (Koizumi and Kollai, 1981). Lateral hypothalamic activation induces sympathetic responses characterized by the tachycardia (Melville *et al.*, 1963), and prevented by C2 spinal cord section and stellate ganglionectomy (Samuels, 2007), whereas the stimulation of the anterior hypothalamus is associated with parasympathetically mediated decrease in HR and BP (Melville *et al.*, 1963). Axons of the neurons in the parvocellular part of the PVN also project to extra-hypothalamic areas of the

brain, such as limbic system, brainstem, and spinal cord where VP and OT influence autonomic functions, including arterial vasoconstriction and increased peripheral resistance (Japundžić-Žigon, 2013). The stimulation of regions of the brain known to induce cardiac reflexes, including limbic cortex, mensencephalic reticular formation, stellate ganglia also generate cardiac effects (Samuels, 2007). The primary brain areas involved in the autonomic component of the brain-heart association include the insula, medial prefrontal cortex and cerebellum (de Morree *et al*., 2013). Stimulation of the right insula increases cardiac sympathetic tone, whereas activation of the left insula is associated with increased cardiac vagal tone (de Morree *et al*., 2013). The clinical importance of the influence of the insula cortices in cerebrovascular events such as stroke is considerable because cardiac arrhythmia in these patients appears to occur more frequently after left insula infarction suggesting sympatho-vagal balance has been impaired (Oppenheimer, 2006). Furthermore, the right hemisphere tends to be predominantly sympathetic, whereas, the left hemisphere is mostly parasympathetic (Oppenheimer *et al*., 1992). There is a strong relationship between short-term rhythmical fluctuations of HR and respiratory activity called respiratory sinus arrhythmia (Larsen *et al*., 2010).

1. 5. Respiratory Sinus Arrhythmia

Respiratory sinus arrhythmia (RSA) is a cardiorespiratory variation between inspiratory R-R interval shortening and expiratory R-R lengthening (Tan and Taylor, 2010; Kabir *et al*., 2013). The inhibition of RSA by antagonists of muscarinic receptors or by vagal cooling (Japundzic *et al*., 1990), suggests vagal drive to be the predominant force mediating RSA (Egizio, 2011), although

sympathetic activation and respiratory frequency may determine the level of its amplitude (Grossman and Taylor, 2007). The increased inspiratory mediated tachycardia is due to hyperpolarisation of cardiac vagal motor neurons which cause the loss of vagal neuronal responsiveness to the baroreflex (Dergacheva *et al*., 2010), and the inhibition of cardiovagal motor neurons by central inspiratory neurons (Frank and Mendelowitz, 2012). This hyperpolarisation of cardiac vagal motor neurons may be related to an increase in activity of slowly adapting stretch receptors, located within larger airways of the lungs, impeding the vagoexcitation baroreflex and chemoreflex (Berntson *et al*. 1993). Therefore, RSA is considered as a modulation of vagal input to the sinus node pacemaker of the heart, rather than being the gate completely inhibiting vagal influence during the inspiration (Egizio *et al*., 2011). Adrenomedullary catecholamines might influence the HR, but the latency of the release of these catecholamines and their relatively long half-life in plasma (about 2 min) are sufficiently long to produce direct manifestations in short-term rhythmical fluctuations (Berntson, *et al*., 1993). Thus, RSA is considered as a non-invasive quantitative index of cardiac vagal activity in humans (Egizio *et al*, 2011). However, central neural or humoral interactions, and mechanical feedback mechanisms are a complex of integrated respiratory and cardiovascular responses that together contribute to the genesis of RSA (Grossman and Taylor, 2007). Furthermore, juxtapulmonary receptors (J-receptors) introduce confounding effects on HR with extreme pulmonary stretch, leading to an increased vagal inhibition (Berntson *et al*., 1993). Increase in respiratory depth

may lead to an increasing peak vagal inhibition which results in a fall in mean vagal control of the heart and an elevated RSA (Berntson *et al*., 1993).

1. 5. 1. Genesis and Mechanisms of RSA

The RSA is directly associated with the interaction between the cardiovascular and respiratory systems, but the amplitude of rhythmic HR fluctuations is greatly dependent upon both the respiratory frequency and tidal volume (Ritz and Dahme, 2006). RSA magnitude under steady-state conditions is inversely proportional to respiration rate and directly associated with tidal volume (Grossman and Taylor, 2007). However, complex interactions between central and peripheral factors, including cardiorespiratory rhythm generators, baroreceptor reflex, chemoreceptor reflex, mechanical and metabolic factors may account in the genesis of the RSA (Garcia III *et al*., 2013), although neural mechanisms overshadow non-neural factors as the RSA tends to be eliminated by autonomic denervation (Berntson, 1993).

1. 5. 1. 1. Central Rhythm Generators

The persistence of HR fluctuations at the approximate respiratory frequency despite the absence of respiration as reported in heart transplant recipients and breath holding or after the elimination of pulmonary reflexes by deafferentation (Anrep *et al*., 1936a, 1936b), suggests that there is a central respiratory generator influencing the HR rhythmicity in the absence of peripheral inputs (Bernardi *et al*., 1989). The brain stem cardio-respiratory generator drives the phrenic efferent neurons and modulates the central outflow, including the respiratory generator, sympathetic generator and central vagal drive (Berntson

et al., 1993). The respiratory generator independent of lung inflation and located in both the dorsal respiratory group and ventral respiratory group (Loewy and Spyer, 1990), inhibits vagal motor neurons during inspiration, but mildly activates them during expiration (Berntson *et al.*, 1993). However, the sympathetic generator located in the lateral medulla, ventral medulla and raphe nuclei (Loewy and Spyer, 1990), although driven by baroreceptor inputs (Kabir *et al.*, 2013), produces an intrinsic rhythm which persists despite baroceptor deafferentation (Berntson *et al.*, 1993). There is a reduction in baroreflex resetting generated by direct hypothalamus or amygdala stimulation during a normal response to stress (Koizumi and Kollai, 1981). Adrenergic blockade increases RSA amplitude (Taylor *et al.*, 2001), and mental tasks reduce RSA by increasing the sympathetic tone (and concomitantly decreasing parasympathetic outflow) that reduces fluctuations in HR at the respiratory frequency (Bernardi *et al.*, 2000), indicating the presence of cardiac sympathetic efferent neurons in the modulation of RSA (Sin *et al.*, 2010). The phasic central cardiac generator, associated with the tonic brain stem excitatory interference (known to have a reticular origin) conduct a huge excitatory input to sympathetic motor neurons in the intermediolateral cell column of the spinal cord (Berntson *et al.*, 1993). The sympathetic motor neurons are activated by inspiration, but mildly inhibited during expiration (Berntson *et al.*, 1993). Finally, the central vagal drive (excitatory vagal drive only) elicits tonic drive on vagal motor neurons and is associated with a variety of centres which enhance vagal motor activity, such as the anterior hypothalamus (Porges, 2009). The suprabulbar system may reset the baroreflex mechanisms under stress

conditions, increasing BP and HR, but decreases the amplitude of RSA (Berntson *et al.*, 1993).

1. 5. 1. 2. Peripheral Factors in the Genesis of RSA

The decrease in intra-thoracic pressure during inspiration due to the descent of the diaphragm, induces an increase in the pressure of the right atrium due to increased venous return, which activates the atrial stretch receptors (Bainbridge reflex) capable of eliciting a small portion of cardiac acceleration during the inspiration (Larsen *et al.*, 2010). This increased blood flow into the thoracic vena cava during the inspiration (Berntson *et al.*, 1993), with a corresponding increased HR, raises the right ventricular stroke volume, leading to increased pulmonary perfusion which enhances the ventilation-to-perfusion ratio, beneficial for gas exchange (Berntson *et al.*, 1993). There is also a corresponding fall in left ventricular stroke volume (Berntson *et al.*, 1993), which induces a decrease in arterial BP and resets the baroreflex mechanisms, leading to increased HR (Schreihofer and Guyenet, 2003). Spontaneous BP fluctuations (due to respiration) are generally mechanically generated and elicit variations in venous return and cardiac output (Wise *et al.*, 1981). Therefore, the inspiratory mediated tachycardia helps to compensate the fall in left ventricular stroke volume in order to maintain normal cardiac output. Nevertheless, the contribution of this mechanical component is still controversial (Karemaker, 2009a) because the latency from the moment BP changes to when the next P wave occurs is too short to contribute to the genesis of RSA (Eckberg, 2009). Among peripheral components contributing to the RSA genesis, the baroreceptors and chemoreceptors are the most powerful

vagal excitatory tools which are inhibited during inspiration (Hayano *et al.*, 1996). However, there is evidence that breathing frequency and tidal volume have influence on RSA (Ritz and Dahme, 2006). Within a range of normal breathing (6-30 breaths/minute), faster HR and lower TV are associated with a decreased RSA, almost in a linear fashion (Ritz and Dahme, 2006), whereas fast and shallow breathing is accompanied with less vagal modulation than slow and deep breathing (Egizio *et al.*, 2011). This may be due either to medullary cardiac-respiratory control, or to peripheral factors (Ritz and Dahme, 2006). The slower respiratory rate and greater relative decreased negative intrathoracic pressure during inspiration allow more time for vagal stimulus to be expressed on the sinus node and to facilitate the venous return which leads to an increase in pulmonary capillary perfusion with alveolar ventilation (Sin *et al.*, 2010). Slow and deep breathing is associated with proportionally greater alveolar ventilation than rapid, shallow breathing (Ganong, 2005). Although controlled breathing increases RSA (Bernardi *et al.*, 2000; Cysarz and Bussing, 2005), when the frequency becomes closest to spontaneous rate, RSA does not show significant changes in RSA magnitude (Stark *et al.*, 2000; Pinna *et al.*, 2006). Contrarily, at low breathing frequency, there is an acceleration of the heart (Pinna *et al.*, 2006) which may not be sympathetically mediated as adrenergic blockade failed to change the pattern of the cardiac acceleration during the inspiration (Taha *et al.*, 1995). The modulation of the frequency-dependent respiration-RSA phase relationships and changes in the respiration-RSA relationships across different breathing frequencies reflect changes in cardiac vagal activity due to central and/baroreflex interactions (Sin *et al.*,

2010). Furthermore, old age is associated with changes in sympatho-vagal balance, with a significant increase in sympathetic activity (De Meersman, 1993), which exhibits a significant mean level decrease in RSA (Hinnant *et al.*, 2011), explaining a relative stable RSA through infancy and earlier childhood (Hrushesky *et al.*, 1984). During supine rest, HR and BP are lower as the body is in a relaxed state. From supine position (a state of high parasympathetic activity and low sympathetic activity) to standing, there is a withdrawal of parasympathetic activity and a concomitant increase in sympathetic activity (Mourot *et al.* 2004). Upright posture appears to unload arterial baroceptors probably due to decreased pressure in sino-aortic structures as tendency of the blood leaving the left ventricle is to flow downward and diminishes the baroreflex drive on vagal motor neurons, inducing a reduction in RSA amplitude (Papegaaij *et al.*, 2014; Saul *et al.*, 1991; Berntson *et al.*, 1993). In addition, passive head-up tilt is known to increase the HR and vascular resistance (Tahvanainen *et al.*, 2009), increases sympathetic tone and decreases vagal motoneuron responsiveness to stimulatory inputs (Cooke *et al.*, 1999). Contrarily, during the predominant parasympathetic state (supine position), the pressure in the sino-aortic structure increases due to more blood reaching the sino-aortic structures, the baroreceptor reflex resets to decrease the HR and BP and the respiration-RSA phase relationship does not change with breathing frequency (Mourot *et al.* 2004). Synchronised sleep reduces sympathetic activity with a decreased cardiorespiratory interaction leading to reduced RSA (Berntson *et al.*, 1993). Non-rapid Eye Movement (NREM) is associated with increased HF power, indicating an increased RSA in adults (Vanoli *et al.*, 1995), whereas

sleep deprivation induces an excessive activation of cardiovascular and sympathetic nervous system which reduces RSA (Zhong *et al*., 2005). Both central respiratory generator and pulmonary stretch receptor afferent neurons can modulate the sensitivity of the brain stem networks which control vagal cardio- motor outflow (Ritz and Dahme, 2006). However, the central respiratory generator appears to be the more predominant driver of RSA in the intact individual with normal low volume respiration where lung inflation receptors are less effective in gating vagoexitatory afferent inputs (Berntson *et al*. 1993). Contrarily, during a high respiratory volume, juxtapulmonary receptors exert an inhibition on the central respiratory generator, thus pulmonary feedback might has predominant role in the genesis of RSA (Taylor *et al*., 2001).

1. 5. 1. 3. Other Determinants of RSA

There are other determinants of RSA, including polyvagal and neurovisceral theories. The polyvagal theory indicates that the efficient RSA flexibility is linked to both the number of myelinated vagal fibres and the ratio of myelinated and unmyelinated vagal fibres (Porges, 2009) described earlier (section 1.4.1). The vagally mediated HR reductions that are not associated with RSA increase originate from the DMN, whereas changes in RSA magnitude are coming from the NA (Grossman and Taylor, 2007) (see section 1.4.1). However, the neurovisceral theory shows that the prefrontal cortex exerts an inhibitory influence on sub-cortical structures to allow the organism to flexibly regulate its behaviour, in response to changing environmental demands (Thayer, 2006). For instance, the amygdala which is under tonic inhibitory control from the prefrontal cortex (Porges, 2009), becomes active during threat because of the

hypoactivation of the prefrontal cortex associated with sympathoexcitatory circuits (Porges, 2009). This inhibitory process can be indexed by measures of vagal function such as the RSA magnitude quantified by HRV parameters (Thayer and Lane, 2009). Several types of anxiety disorders, including social, post-traumatic stress have their amygdala hyper-responsiveness to a variety of affective challenges (Thayer *et al.*, 2012). This theory shows that disruption of vagal inhibitory function might contribute to emotion-related activation of proinflammatory pathways (Tonhajzerova *et al.*, 2013).

1. 5. 1. 4. Physiological Significance of RSA

The increase in systolic blood pressure (SBP) within a given beat stimulates the baroreflex arc to adjust the timing of the following R-wave such that the diastolic pressure is stabilized (Karemaker, 2000). The reduced RSA following baroreflex denervation and the corresponding increased arterial blood pressure in animal model indicates that RSA acts to buffer BP fluctuations (Tang and Dworkin, 2009). In addition, RSA matches the perfusion of the lungs to the ventilation during each respiratory cycle, (Hayano and Yasuma, 2003), enhances the oxygen uptake (Larsen *et al.*, 2010), and improves both energy efficiency in pulmonary circulation and perfusion-ventilation efficiency (Hayano *et al.*, 1996). Therefore, heart failure patients are advised to have slow breathing in order to improve their pulmonary gas exchange efficiency (Joseph *at al.*, 2005; Raupach *et al.*, 2008).

1. 5. 2. Measurement of RSA

The response of the ANS outputs of the central autonomic network through the SA node generates heart rate variability (HRV) used to assess cardiac autonomic regulation through quantification of sinus rhythm variability (Thayer and Lane, 2000). HRV refers to a measure of variations between consecutive heart beats to elicit quantitative rhythms that give a quantitative non-invasive measure of autonomic modulation of cardiac activity (Garcia *et al*., 2013), and is measured in the time and frequency domains (Dantas *et al*., 2010) to provide an insight into neural control mechanisms of the heart (Stein and Pu, 2012). To obtained reliable measures of HRV, QRS complexes have to be of good quality for the accuracy of R-peak detection, sampled at the range between 250 Hz and 1 KHz for adequate time domain HRV analysis, with appropriate filtering in order to remove baseline fluctuation and high-frequency noise and to discount ectopic beats and artefacts (Stein and Pu, 2012), and its reduction indicates impairment of the ANS (Kleiger *et al*., 2005). Nevertheless, short-term HRV ranging from 0.5 to 5 minutes is mostly related to the oscillations in vagal neural traffic, driven by rapid changes in ACh (Tonhajzerova *et al*., 2013), the sympathetic action being too slow to be involved in rapid beat-to-beat changes (Thayer *et al*., 2012). The commonly used quantitative measure of parasympathetic tone deriving from ECG recording is high frequency (HF) spectral power (0.15–0.4 Hz) suggested reflecting changes in parasympathetic control and associated with respiratory rhythm (Egizio *et al*., 2011). Alternatively, the peak-to-trough (peak-valley) method provides a common time-domain index of RSA which is extracted by subtracting the minimum HR

during expiration from the maximum HR during inspiration within the respiratory cycle (Egizio *et al.*, 2011). This time domain analysis extracts the amplitude of HR fluctuations related to each breathing cycle such as the difference between the fastest HR during inspiration and the slowest HR during the expiration (Ritz and Dahme, 2006).

1. 5. 2. 1. Time Domain Indices

Time domain analysis addresses how much variability is in the HR by measuring time differences in R-R interval (Petkovic *et al.*, 2013). Time domain parameters are easily extracted with statistical methods managed on the set of adjacent intervals even from a short time recording (5-10 minutes) (Petkovic *et al.*, 2013). Standard HRV indices were calculated according to the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, including the PNN50 (%) and RMSSD (msec), both reflecting the cardiac parasympathetic activity (Stein and Pu, 2012). They represent the proportion of differences in consecutive RR intervals that are longer than 50 ms and the root mean square of successive RR differences respectively (Malik, 1996)

1. 5. 2. 2. Frequency Domain Components

Frequency domain methods are used to divide the total variance of the heart rate into the variance accounted for by underlying groups of frequencies (Petkovic, 2013) and tend to quantify the underlying rhythms (Pitzalis *et al.*, 1996). Frequency domain analysis uses analysis of frequencies of R-R interval oscillations (the pertinent one in RSA being oscillation in R-R interval at the

frequency of ventilation). The spectral frequency domain analysis can be performed by the Fast Fourier Transform (FFT) method to avoid losing some information regardless of its limited frequency resolution which is affected by both the duration of the recording and the windowing process (Stein and Pu, 2012). FFT is a mathematical operation used in spectral analysis, involves conversion of a time domain to frequency domain function, and allows to efficiently estimating component frequencies in data from a discrete set of values sampled at a fixed rate (Edelman *et al.*, 1999). Several indices can be extracted from the FFT, including high frequency (HF) component (0.15 to 0.40 Hz) which reflects respiratory rhythm and is associated with parasympathetic control of the HR, low frequency (LF) component (0.04 to 0.15 Hz) which reflects the baroreflex mechanism, and represents mixed parasympathetic and sympathetic activities useful in short-term spectral recordings (5 to 10 minutes) (Petkovic *et al.*, 2013). Contrarily, ultra-very low frequency (UVLF) component with the band below 0.003 Hz represents a long-term recording (Larzen *et al.*, 2010). Neuroimaging studies show more evidences of specific brain areas such as the prefrontal cortex, anterior cingulate cortex and insula, associated with HF-HRV (Lane *et al.*, 2009), indicating that the cardiac vagal function indexed by RSA reflects the central-peripheral nervous system integration (Porges, 2009). The LF/HF ratio is calculated to express the balance between sympathetic and parasympathetic modulation (Kleiger *et al.*, 2005; Cysarz *et al.*, 2012). LF and HF frequency powers of HRV can further be represented in normalised units (nu) which are calculated by dividing the frequency power by the total frequency power minus the very low frequency (Task Force, 1996).

RSA evaluation using HRV spectral analysis is sufficiently sensitive to detect cardiovagal regulation related to the acute mental stress (Tanhajzerova *et al*, 2013) so that stressors are often associated with an increase in LF (centred around 0.1Hz) power, a decrease in HF (0.12 or 0.15-0.4 Hz) power and/or an increase in LF/HF ratio (Berntson *et al*., 1994).

1. 6. QT Interval

The QT interval used as an index of cardiac sympathetic activities is an electrocardiographic exhibition of ventricular depolarisation and repolarisation which undergoes subtle beat-to-beat fluctuations (Conrath and Opthof, 2006). The presence of sympathetic and parasympathetic fibres on atrio-ventricular muscles, pacemaker and conducting tissues (Ulphani *et al*., 2010) indicates that QT interval is strongly influenced by the cardiac ANS. Therefore, changes in ANS efferent activity may influence cardiac depolarisation and repolarisation and alter the QT interval (Taubel *et al*., 2012). However, the duration of QT interval may also be influenced by different factors, including circulating catecholamines and HR frequency (Arrowood *et al*., 1993). Nevertheless, experiments assessing HR-QT relationship during cold pressor and valsava manoeuvres indicated a significant increase in HR, contrasted with a minimal change in QT interval in normal human subjects (Arrowood *et al*., 1993), indicating that manoeuvres that increase cardiac sympathetic activity may not induce changes in QT interval. This is because the sympathetic discharge to the ventricle may be minimal as the sympathetic fibres that regulate HR have different origins from those modulating the QT interval in humans and dogs (Arrowood *et al*., 1993). Ablation of right stellate ganglion in dogs resulted in a

significant reduction in HR from 116 ± 35 beats/min (control) to 90 ± 17 beats/min, $p<0.05$, whereas the ablation of left stellate ganglion did not show a significant change 113 ± 8 (control) to 120 ± 10 beats/minutes in dogs (Schwartz and Stone, 1979). After maximal treadmill exercise, HR increased only from 90 ± 17 beats/min to 160 ± 10 beats/min with right ablated stellate ganglion, whereas dogs with left ablated stellate ganglion showed an increased HR from 120 ± 10 beats/min to 258 ± 6 beats/min, almost similar to control dogs which showed an increase in HR from 113 ± 8 to 243 ± 10 beats/min. These observations were consistent with the results found by Jonnesco (1921) who indicated that removing the left stellate ganglion in a patient with angina pectoris produced a marked increase in HR which was still evident several years after surgery. Therefore, the sympathetic control of HR is mediated mainly by fibres from right stellate ganglion, whereas QT interval is mainly influenced by impulses from the left stellate ganglion (Schwartz and Stone, 1979) because left stellate influence is predominant over the posterior wall of the ventricles, while right stellate controls more the anterior ventricular walls (Vaseghi *et al.*, 2012).

1. 6. 1. QT Interval Modulation

The cardiac action potential is modulated by different ion channels, including sodium, potassium, and calcium ion channels (Couchonnal and Anderson, 2008) and the impairment of these ions channels affect the QT interval (Sirker and Shan, 2010). An increase in potassium ion conductance which induces an earlier repolarisation reduces the QT interval (El-Sherif and Turitto, 2011; Zhao *et al.*, 2012), whereas a reduction in potassium ion current observed in

ventricular hypertrophy leads to the delayed repolarisation with a corresponding long QT interval (El-Sherif and Turitto, 2011). An abnormal lengthening of depolarisation can be a consequence of some physiological abnormalities, including an abnormal increased inflow of calcium ions, and an abnormal decrease in potassium ions outflow (Al-Khatib *et al.*, 2003; Jeony *et al.*, 2012). Therefore, the acceleration of calcium channel reactivation during an increase in sympathetic activity may induce an early after depolarisation characterized by a long QT interval (>440ms) (Schwartz *et al.*, 1993).

1. 6. 2. QT Interval and the Influence of Catecholamines

In ischaemic heart disease patients, an increased HR of 100 bpm caused by atrial pacing would produce a QT interval decreased of approximately 66 msec, contrarily to an increased HR of 100 bpm generated by exercise which would induce a QT interval decreased of about 95 msec (Rickards and Norman, 1981). The shortening in QT interval due to paced HR similar to HR achieved with exercise was less shorter than the one related to exercise (Fananapazir *et al.*, 1983), suggesting that there is more than an increase in HR in the QT interval reduction. QT shortening may be due either to an increase in local release of adrenergic neurotransmitters in the myocardium or to an increase in circulating catecholamines (Arrowood *et al.*, 1993). However, reflex manoeuvres which induce little or no circulating catecholamines (duration and level), such as Valsalva manoeuvre and cold pressor testing showed minimal changes in QT interval shortening and moderate increase in HR (Stratton *et al.*, 1983), indicating that the duration of QT interval is only partly influenced by the HR (Davidowski *et al.*, 1984). Therefore QT modification (reduction) is mostly

related to catecholamines circulating instead of local adrenergic neural stimulation to cardiac muscle (Brath *et al.*, 1992; Quigg *et al.*, 1989). An abrupt increase in HR after a bolus injection of Isoproterenol generates only a little change in QT interval (Opthof *et al.*, 1991; Lecoq *et al.*, 1989), while a continuous intravenous infusion of the same product induces a significant decrease in QT interval (Kawataki *et al.*, 1984), suggesting that a short duration of cardiac stimulation during these reflex manoeuvres may be associated with the lack of QT shortening (Arrowood *et al.*, 1993). A prolonged cardiac sympathetic stimulation (30 sec-5 min) associated with higher concentration of noradrenaline in ventricular muscles and Purkinje neurons, shortens cardiac action potential and induces a sympathetically mediated shortening in QT interval (Kass and Wiegers, 1982; Arrowood *et al.*, 1993). Contrarily, a short duration in cardiac sympathetic stimulation (1-3 sec), inducing lower noradrenaline concentration and a prolongation of cardiac action potential generates a lengthening in QT interval (Arrowood *et al.*, 1993). A greater cardiac sympathetic stimulation without parasympathetic withdrawal (using isoproterenol for instance) induces less shortening in the QT interval as HR increases, compared with the QT interval generated by the blockade of cardiac parasympathetic nerves via atropine administration (Magnano *et al.*, 2002). Therefore, different autonomic and physiological reflexes which induce an increase in HR may give different changes in QT intervals. The autonomic influence on the QT interval operates in two ways: either directly through an action on the ventricular myocardium or indirectly via associated changes in HR and accompanying effects of HR on the QT interval (Magnano *et al.*, 2002). The

prolongation of action potential duration associated with a lower concentration of noradrenaline occurs on α_1-stimulation in the presence of β-blockade (Giotti *et al.*, 1973), whereas the shortening of action potential duration with higher concentrations in ventricular muscle and Purkinje fibres (Kass and Wiegers, 1982; Quadbeck and Reiter, 1975) occurs as a consequence of β_1- and β_2-stimulation (Dukes and Vaughan Williams, 1984). This is because the time-dependent outward potassium ion current is associated with higher levels of noradrenaline (Arrowood *et al.*, 1993). Therefore, brief nerve stimulation associated with lower concentration of noradrenaline may lengthen action potential duration by preferentially stimulating α_1-receptors (Arrowood *et al.*, 1993), whereas prolonged adrenergic stimulation (consequence of higher noradrenaline concentrations β_1- and β_2-receptors stimulation) shortens action potential duration (Apkon and Nerbonne, 1988). In general, the modulation of QT interval depends on the duration and uniformity of cardiac adrenergic stimulation (Arrowood *et al.*, 1993).

1. 6. 3. QT interval Correction and Measurements

The assessment of QT intervals cannot readily distinguish between the effects of HR changes, autonomic effects, and effects induced by ion channel blockade (Taubel *et al.*, 2012). Various mixtures of reflexes involved in the increase in HR might influence the relationship between RR interval and QT interval, even in the same individual when subjected to different HR stimuli (Benatar and Decraene, 2001). Due to its inverse relationship to the HR, the measured QT interval is generally corrected by means of various formulae to a less heart rate dependent value known as the QTc interval. The QTc interval (as accepted by

the International Conference on Harmonisation (ICH) E14 guideline) is preferred because it removes factors that may lead to a false signal during the studies (Taubel *et al.*, 2012). There are two different methods of measurement of the QT interval: The threshold method (figure 4) and the tangent method (figure 5) (Panicker *et al.*, 2009).

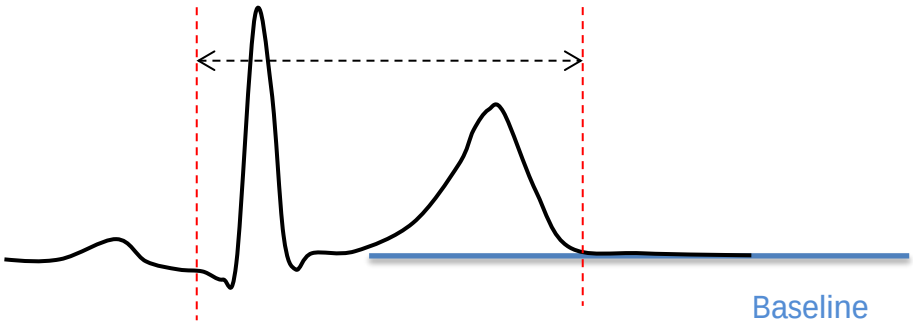

Figure 4: Threshold method. T wave offset is determined by the point at which the T wave reaches the isoelectric baseline (Arrowood *et al.*, 1993).

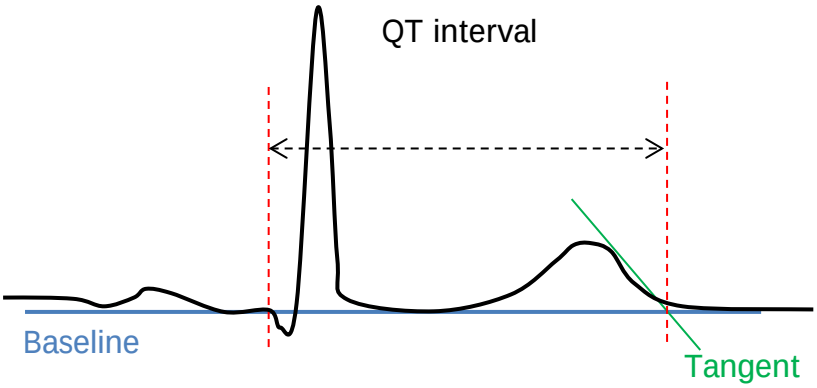

Figure 5: Tangent method. The QT interval is defined as the distance from the beginning of QRS to the intersection of a tangent to the steepest part of the descending portion of the T wave with the ECG baseline (Arrowood *et al.*, 1993).

The QT interval obtained by the tangent method is reported to be up to 10 milliseconds shorter, leading most of people to use the threshold method

(Panicker *et al.*, 2009). In addition, the comparison of QT intervals between different people with different heart rates requires also an application of QTc interval which allows comparison of QT interval values over time at different HR (Davey, 1999). The QT-RR relationship is considered as a reproducible description of cardiac repolarisation over time and the dependence of QT interval on HR seems to be an intrinsic electrophysiology property of the ventricular myocardium (Panicker *et al.*, 2009). The practical meaning of QTc interval measurement depends on the correction formula used (Aytemir *et al.*, 1999). The four most used formulae accepted to be taken from a lead II ECG (Benatar and Decraene, 2001) are presented in the table 2, each of them having some weaknesses in their application because different autonomic and other physiological reflexes that increase HR influence directly the depolarisation (Aytemir *et al.*, 1999). The relationship between the RR and QT interval is unlikely to be constant even in the same subject when exposed to different HR changing stimuli. However, QTc interval seems to be normal at QT interval estimated at the HR of 60 beats per minute and QTc longer than 0.44 seconds may be considered as abnormal (Jeony *et al.*, 2012).

Method	Formula	Comments
Bazett	$QTc = QT/\sqrt{RR}$ interval (sec)	The easiest to remember, widely used (Benatar and Decraene, 2001), and most criticised as it gives a slight over correction at higher HR (Milne *et al*., 1982).
Hodges	$QTc = (QT + 1.75$ [rate - 60])	Have more uniform rate correction over a wide range of HR and is gradually gaining more widespread acceptance (Kumar *et al*, 2004).
Fredericia	$QTc = QT/\sqrt[3]{RR}$	Widely used, gives more consistent results at fast heart rates. May lead to an under correction at peak exercise. It dissociates changes in HR most effectively (Tabo *et al*., 2006).
Framingham	$QTc = QT + 0.154 (1-RR)$	Have more uniform rate correction over a wide range of heart rates, but criticised as the Fredericia correction (Sagie *et al*., 1992)

Table 2: QT-HR Correction Formula. QT interval measured in msec and R-R calculated in sec. During this study where subjects were not doing any exercise, Fredericia method was used (see QTc interval, section 2.3).

1. 7. General Aim

This study aims to investigate whether, and to what extent the stimulation of various gastric receptors may influence cardiovascular changes in young healthy subjects. Although different studies have indicated the concomitant activation of the two branches of the ANS after of water drinking (Brown *et al.*, 2005; McHugh *et al.*, 2010, May and Jordan, 2011), little is known about the role of cold water on the autonomic cardiovascular balance. Therefore, the present study will determine whether the cold temperature combined to the hypo-osmotic properties of water play a role in the cardiovascular responses to drinking in normal subjects. The use a small volume of water (such as 300 mL) will determine whether the cardiovascular responses to such volume of water will be the same as the ones induced by larger volume of about 500 mL of water described in other studies (Brown *et al.*, 2005; McHugh *et al.*, 2010). Small quantity of water ingestion may be expected to activate the GI osmoreceptors, whereas a larger volume may be speculated to activate both the local osmoreceptors (GI receptors), the osmoreceptors located in the portal system, and/or the central osmoreceptors located in the circumventricular organs (McKinley *et al.*, 1992) and NTS (Daniels and Fluharty, 2004). We will also compare the beat-to-beat cardiovascular responses and autonomic efferent modulation to water with the responses to a same volume of physiological (0.9% w/v) saline solution ingested at different temperatures. This will allow investigating if the pressor effects reported after water ingestion (Brown *et al.*, 2005) were not related to volume loading effect as saline drinking is expected to induce a greater plasma volume than water and to assess the role of cold

temperature on these effects. A further contributory mechanism could be evoked by comparing the influence of gastric distension and gastric distension combined to gastric cooling on the cardiovascular autonomic efferent activities. The cardiovascular responses to gastric stretch or gastric distension and gastric cooling will determine if the known pressor effects after water drinking were associated with gastric distension and to investigate to role of gastric cooling of the reported increased sympathetic activity after gastric stretch (Van Orshoven *et al.*, 2004). The cardiovascular responses to enteric-coated capsule of peppermint oil ingestion known to activate TRPM8 gastric cold receptors via the release of its menthol content (Papathanasopoulos *et al.*, 2013) will differentiate the cardiovascular responses to cold effects only and the cardiovascular responses to cold combined to other gastric stimuli such as gastric stretch or water hypo-osmolality. Finally, there are conflicting opinions expressed as to the adequate reproducibility of HRV parameters in both the time and the frequency domain parameters when assessing cardiac autonomic activities in the longitudinal studies where different individuals are recorded over a time and during different sessions (Van boven *et al.*, 1998; Lord *et al.*, 2001). Various external factors, including noise and neuropeptides released during long ECG recording may exert an inhibitory effect on cardiac vagal tone (Bernardi *et al.*, 1992) and disrupt the vagal control on the heart at rest. Therefore, the assessment of the time dependent effects on and reproducibility of HRV parameters and QTc interval when quantifying cardiovascular autonomic efferent activities will be investigated without a drink to determine potential cardiovascular autonomic variations associated with the time spent during the

recording period or with the second exposure to the protocol procedures in our laboratory.

CHAPTER 2. GENERAL METHODS AND MATERIALS

2. 1. Study Population

Healthy non-smoking and normotensive subjects (aged between 18 and 45 years) were recruited to participate voluntarily in different experiment sessions evaluating the effects of gastric autonomic afferent activity on cardiovascular autonomic efferent balance. The BP was checked at the beginning and the end of each ECG recording period using a brachial electronic sphygmomanometer (boso-medicus, Bosch+Sohn, Germany) to make sure subjects were normotensive (BP ≤ 130/85 mmHg) and to assess any changes which could occur during the recording period (Kshirsagar *et al.*, 2006). None of the subjects were taking any medication affecting autonomic regulation or the cardiovascular system and none had a history of gastro-intestinal disease. They were not elite athletes as these individuals show little or no modulation of their parasympathetic autonomic function (Blasco-Lafarga *et al.*, 2013). All subjects were requested to avoid sleep deprivation 24 hours prior to the experiment session as this has been shown to induce excessive activation of the sympathetic nervous system which reduces RSA (Zhong *et al.*, 2005). A modulation at any point in the reflex arc of the cardiovascular ANS can alter end-organ function and can be influenced by many factors, such as eating, drinking coffee, smoking, ethanol, and stress (Tannus *et al.*, 2013). Therefore, subjects were asked to avoid alcohol at least 24 hours preceding the experiment session and caffeine the day of the recording because both alcohol and caffeine have been documented to provoke anxiety disorders capable of impeding normal vagal inhibitory control on the heart (Thaler *et al.*, 2012), as

described in the general introduction. In addition, the exposure of the luminal surface of the stomach to irritant chemicals or prior heat stimulation leads to sensitisation of responses to gastric distension (Ozaki, and Gebhart, 2001), and food and water drinking are reported to induce different cardiovascular effects, including cardiac vagal activation in response to water ingestion (Brown *et al.*, 2005), cardiac sympathetic stimulation due to gastric stretch (Van Orshoven *et al.*, 2004). Therefore, subjects were requested to avoid food and drink ingestion 2 hours prior to recording sessions.

2. 2. Experimental Protocol

Each protocol was approved by the University of Wolverhampton Life Sciences Ethics Committee and written informed consent was obtained from each subject. All experiments were performed in the morning (between 08:00 and 11:00 am) in order to minimize the experimental impact of the variation in the baseline autonomic function known to occur over the course of the day (Akerstedt and Gillberg, 1983). Subjects were asked to empty their bladders before the experiment session as bladder distension is known to evoke an increase in the sympathetic nervous system and BP (Fagius and Karhuvaara, 1989; Mark *et al.*, 1985). This also prevented subjects from discomfort and interruption of the experiment session while recording if a subject has to empty the bladder. They were asked to wear suitable clothing to ensure their comfort and to facilitate the use of recording equipment. Subjects attended an hour of initial habituation and training visit performed in one of the human research laboratories at the University of Wolverhampton where all experiment sessions were performed. During this visit, subjects were trained to breathe to a timer

operating at 0.2 Hz, so setting the ventilation to 12 breaths per minute, an estimated breathing frequency commensurate with normal physiological resting respiratory frequency (Littleton, 2012). The study assessing HRV and spontaneous baroreflex sequences using the frequency controlled breathing procedure in young healthy subjects requested to the participants to breathe at a frequency of 12 breaths/minute, which avoided artefacts in the LF range of HRV from irregular slow breaths (Valipour *et al.*, 2005), and better separated the peaks of spectral power of the low and high frequency band (Lobnig *et al.*, 2003). Subjects rested comfortably on a therapy table in a semi-supine position, in a quiet and well ventilated room as noise is known to activate the sympathetic nervous system by disrupting vagal inhibitory function (Pizalis *et al.*, 1996). This position increases the cardiovagal control that could counteract the pressor effects of sympathetic activation (Routledge *et al.*, 2002). Subjects were fitted with a lead II arrangement of a standard 3-lead ECG using hypoallergenic electrodes and gel. They were also fitted with a respiratory belt transducer (UFI 1132 Pneumotrace II ^{TM}, ADInstruments, Australia) directly connected to the PowerLab (4/25 T, ADInstruments, Australia) in order to assess their respiratory movements (Bhaskar *et al.*, 2013), and their ability to breathe to the online set up beep (corresponding to 0.2 Hz) and correct them if necessary during the experiment session. Continuous BP was monitored using finger plethysmograph (Ohmeda 2300, Finapres Medical Systems, USA) from the middle finger of the left hand. The arm was covered with a thick blanket in order to avoid a cooling and vasoconstriction of the finger (Jagomagi *et al.*, 2001) during the recording period and the upper body was also covered with a

light blanket to prevent the whole body from cooling down. Subjects were asked to observe a period of stabilisation (20 minutes at least) where subjects were lying quietly and relaxed on a therapy table, as any physical activity could interfere with the normal parasympathetic control of the ANS in healthy people (Sin *et al.*, 2010). The cardiac parasympathetic withdrawal which reduces arterial baroreceptor reflex sensitivity during exercise (Ogoh *et al.*, 2005) will be expected to be reintroduced during the 20 minutes of acclimatisation period. This was followed by a 5 minute recording session prior to drinking (baseline or point zero). They were then asked to drink different solutions including, Ispaghula husk solution (to distend the stomach wall), isotonic saline solution (to test volume loading effect), water (to investigate to effect of hypo-osmolality), and peppermint coated capsules (to stimulate gastric cold receptors) followed by various recording intervals during the post-drink period in order to test the cardiovascular. The drinks were served at either 37°C (body temperature) or 6°C (cold temperature) and were ingested within 5 minutes according to a crossover study, as subjects had to drink the solution at either 37°C (control) or 6°C during the first of the two visits according to the experimenter design in order to prevent the effects of habituation responses of the subjects to the protocol not interfering with the temperature. The second visit was done during a separate study visit at least 7 days later. Subjects drank the solution using their right hand without changing their position (Tahvanainen *et al.*, 2009). Immediately after the ingestion, a continuous 20 minute recording session was made as major cardiovascular changes could occur during the first 20 minutes before the solution starts to enter the duodenum (Sun *et al.*, 1988).

This was followed by alternative periods of 5 minutes rest and 5 minutes recording sessions for the rest of the first hour and then with alternative periods of 10 minutes rest and 5 minutes recording during the second hour (figure 6). During the drinking period, the ECG and continuous BP recordings ceased and immediately restarted after finishing the drink. Any sensations of discomfort, bloating, nausea or pain during either the drinking period or the recording time were advised to be reported so that the experiment session could be discontinued. A 5 minute recording period was designed in agreement with HRV guidelines in order to obtain at least 256 R-R intervals which gives the most stable data for RSA analysis (Task Force, 1996).

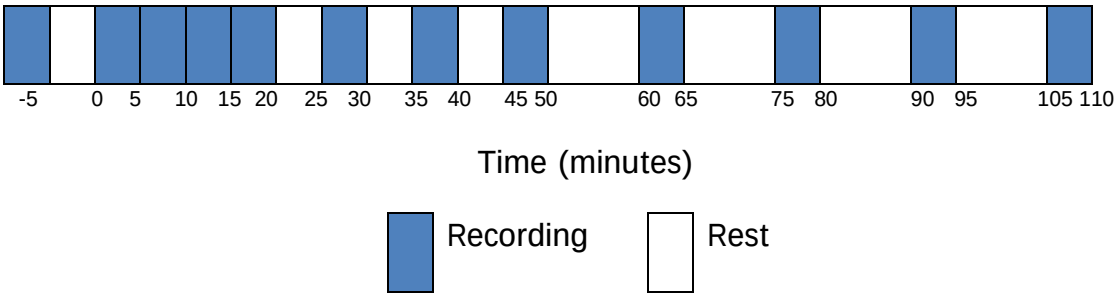

Figure 6: Timeline scale of the recording. Minus 5 reprents baseline recording made prior to the drinking. The ingestion was made at point zero (0) followed by a continuous 20 minute recording session. The rest of the first hour was made by alternative periods of 5 minutes rest and 5 minutes recording sessions. During the second hour, alternative periods of 10 minutes rest and 5 minutes recording sessions were also performed.

However, experiments undertaken in healthy subjects during the time-dependent effects on the CVS and reproducibility of data assessment with no drink (chapter 3), alternative periods of 5 minutes recording sessions and 15 minutes rest over the time course were performed. These experiments aimed to assess cardiac autonomic variations that may occur over the time course on

subjects exposed to a recording process and to monitor the influence of a second exposure to the protocol procedures on HRV parameters and QTc interval values.

2. 3. Measurements

The ECG was recorded and digitised using a sampling frequency of 1kHz (PowerLab 4/25 T and software Chart 5, ADInstruments, Australia) and high-pass filtered at 1 Hz in order to remove low frequency isoelectric line variation which could have had an impact on the software ability to accurately assess R-R intervals (RSA) and for better data analysis (de Castro *et al.*, 2014). Respiratory movements were recorded using a respiratory belt transducer strapped on the chest or at the upper abdominal level used as an immediate *ad hoc* assessment of respiration rate. This position was optimised for reliable recording in each subject. The left hand with plethysmograph cuff rested on a pillow which was positioned at heart level on the table to eliminate the influence of gravity on the observed BP (Girona *et al.*, 2014). The suitable heart-hand height was determined by comparing the mean arterial BP value of the Finapres with the mean arterial BP value of at least two measurements with sphygmomanometer (Van Orshoven *et al.*, 2004).

Heart rate variability (HRV)

HRV analysis attempts to assess cardiac autonomic regulation via quantification of sinus rhythm variability and the sinus rhythm time series being derived from QRS to QRS (RR) interval sequence of the ECG, by extracting only normal sinus

to sinus interbeat intervals (Garcia *et al*., 2013). Mean values of the time and frequency domains of HRV parameters, including HR (bpm), cardiac interval RMSSD (msec), cardiac interval PNN 50 (%), cardiac interval LF power (nu), cardiac interval HF power (nu), and cardiac interval LF/HF (ratio) were assessed from successive R-R intervals in data lengths of at least 256 (Singh *et al*., 2004). Values are reported as mean (± SEM) for every 5 minutes recorded during the baseline time and over the 105 minutes post-drink period. R-R intervals power spectral densities were calculated with Fast Fourier Transform algorithm obtained using a Welch window in 50% overlapping segments (Tan and Taylor, 2010), to provides a smoothed spectral estimate with clearly outlined peaks in low- and high-frequency bands (Singh *et al*., 2004). The value assigned to the overlap parameter determines how many data segments can be obtained from the original sequence by partitioning the data into several shorter segments (Singh *et al*., 2004). The overlap set up to 50% allows the averaging over three power spectral densities to be obtained in order to reduce the error variance of the power spectral density resolution (Attivissimo and Savino, 2001). The HRV Module, an *add-on* to the Chart software, used a threshold detector to identify the R component from each ECG waveform and generated R-R Interval data and bin size was setup at 10 msec. Beats were automatically distinguished by the HRV software and classified into three groups; normal, ectopic or artefact beats. R-R intervals less than 5 msec and greater than 2000 msec were considered as artefacts, whereas R-R intervals greater than 5 msec and less than 400 msec were considered as ectopic beats. In addition, R-R intervals greater than 1600 msec and less than 2000 msec were also

considered as ectopic beats. Ectopic and artefact beats were excluded from analysis. R-R intervals were considered normal if they were greater than 400 msec and less than 1600 msec. Normalized units (nu) offer a better definition of balance of the two components of the ANS (vagal and sympathetic branches) in different situations (Pitzalis *et al.*, 1996). Changes in cardiac interval RMSSD variability, cardiac interval HF power, and cardiac interval PNN 50 (%) were used to assess cardiac parasympathetic activity. However, cardiac interval RMSSD variability has been suggested to be superior to cardiac interval HF power and cardiac interval PNN50 spectral components of HRV because it is less affected by respiratory frequency and HR rhythm (Penttila *et al.*, 2001), and because of its better statistical properties (Task Force, 1996). The RSA variability peak is within the HF power band range (0.15–0.40 Hz) only at respiratory rate around 15 breaths/minutes, whereas slow breathing leads the variability peak to shift towards the LF power band, and when breathing increases, the upper half of the variability peak is even outside the standard HF power range (Penttila *et al.*, 2001). Oscillations in cardiac interval LF bands appear to be mediated by parasympathetic and sympathetic compenents (Pal *et al.*, 2014), whereas cardiac interval LF/HF ratio quantifies the changing relationship between sympathetic and parasympathetic nerve activity (i.e., the sympatho-vagal balance) (Pagani *et al.*, 1986). Therefore, the sympatho-vagal activation was assessed by low frequency power of cardiac interval variability (LF) and cardiac interval LF/HF ratio, considered to reflect sympatho-vagal stimulation and sympatho-vagal balance of the ANS respectively (Brown et al *et al.*, 2005).

Blood pressure (BP)

The measurement of the BP was performed to establish that subjects were normotensive and to ensure correct Finapres calibration (Van Orshoven *et al.*, 2004). Assessment of BP also helped to determine how changes in BP could be correlated with HR changes and to evaluate potential physiological mechanisms associated with those changes which may include sympathetically mediated vasoconstriction manifested as increased BP. Continuous finger blood pressure (Ohmeda 2300, Finapres Medical Systems, USA) channelled through the Chart 5 PowerLab and digitalised using frequency of 1 KHz obtained during the first continuous 20 minutes, and during every 5 minute session, displayed values for systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and HR. Before the attachment of the cuff, the left middle finger was rubbed by the subject in order to warm up the dedicated area, thereafter the sensor was attached. After securing the cuff around the subject's middle finger, the Finapres calibrates itself as followed; the cuff is inflated below normal blood pressure, then the light intensity is measured over several blood pulses, and the amplitude of the oscillations in light intensity is recorded. Afterward, the cuff pressure below normal BP is increased in small increments. During the subsequent step, the cuff pressure that maximises the amplitude of the oscillations in light intensity is found, and recorded (Birch, 2007). SBP (mmHg) was evaluated using the Blood Pressure *Add-On* LabChart's Data Pad feature to get values automatically from the Chart 5 PowerLab in order to assess vascular pressor effects (Brown *et al.*, 2005).

QTc interval

The QTc interval (msec) was calculated using the Fredericia formula from QT interval obtained by the tangent method, this to evaluate the sympathetic tone over the time course of the protocol procedures (Brown *et al.*, 2005). The Fredericia method was used as it dissociates changes in HR more effectivey in our resting subjects (not excercising), whereas the Hodges method is still gaining widespread acceptance (see table 2). QT interval was obtained as a mean of 9 different QT interval measures obtained 3 at the beginning, 3 at the middle, and the 3 at the end of each 5 minute recording period.

Data were stored anonymously in order to maintain confidentiality. The outcomes of the protocol were analysed offline in the light of the results from the different visits to elucidate the autonomic mechanisms underlying the cardiovascular responses to different gastric stimuli over time.

2. 4. Data and Statistical Analyses

Average values of HR (bpm), cardiac interval RMSSD (msec), cardiac interval PNN 50 (%), cardiac interval LF power (nu), cardiac interval LF/HF ratio, QTc interval (msec), and BP (mmHg) were derived from every 5 minute recording period for each subject during the baseline and post-treatment intervals using Microsoft Excel 2010. These mean values were reported as changes from the average of respective pre-treatment baseline in order to eliminate individual differences, using Microsoft Excel 2010. All values were reported as mean ± SE. Statistical analyses were performed by one-way ANOVA for repeated measures using statistical software [InStat version 6.02 GraphPad Prism Software (San Diego, CA)]. The effects of each drink over time were analysed by comparing values at each time point over the post-drink period with the basal values recorded during the 5 min immediately before drinking. Repeated-measures ANOVA with Bonferroni post hoc testing for multiple comparisons were used to test for changes over time from baseline level. Paired t- test was used to compare the values of corresponding time points of the first and the second visit of the same experiment in order to investigate the effects of habituation to the full experiment protocol. Values were rounded to two decimals using Microsoft Excel 2010, except BP where values were rounded to the nearest whole values because both the sphygmomanometer and the Finapres could not detect the BP values to two decimals. For all tests, $p < 0.05$ was considered statistically significant.

CHAPTER 3: TIME DEPENDENT EFFECTS ON AND REPRODUCIBILITY OF DATA, WHEN QUANTIFYING CARDIAC AUTONOMIC EFFERENT ACTIVITIES USING ANALYSIS OF HRV AND QTC INTERVAL IN HEALTHY YOUNG SUBJECTS

3. 1. Introduction

Heart rate variability (HRV) measurement is a non-invasive tool used to monitor autonomic modulation of cardiac activity and both temporal and spectral measures have been used to evaluate several parameters of the cardiovascular system in healthy subjects (Dantas *et al.*, 2010). Short-term fluctuations in HR occur as a result of changes in ANS modification of SA node function (Pitzalis *et al.*, 1996), expressed as a vagally mediated RSA and assessed using HRV parameters (Thayer *et al.*, 2012). However, the use of HRV parameters to determine the neural autonomic balance directed at the heart especially over a long period (24 h for example) is controversial, because the modulation of heart beats is strongly influenced by environmental conditions, mental stress, and body position (Dantas *et al.*, 2010; Tannus *et al.*, 2013). In longitudinal studies where the same individuals are recorded over a time and during different sessions, the assessment of the reproducibility of HRV parameters may be fundamental in order to monitor cardiovascular variations which may occur over the time course. Nevertheless, available data on time dependent effects when monitoring the cardiac parasympathetic autonomic efferent activity or concerning the reproducibility of time and frequency domain parameters of HRV in healthy people, is limited (Pitzalis *et al.*, 1996; Lobnig *et al.*, 2003). More information comes from patients with cardiovascular or metabolic diseases

(Freed *et al*., 1994). Nevertheless, in healthy subjects, long ECG recordings may be affected by the release of neuropeptides, including NPY which occurs as a result of mental activity or stress and is capable of exerting an inhibitory action on cardiac vagal nerves (Bernardi *et al*., 1992). Opinions have been expressed as to the adequate reproducibility of HRV parameters in both the time and the frequency domain parameters (Van Boven *et al*., 1998; Lord *et al*., 2001), especially when different subjects have been recorded in different conditions and using different analytical procedures of data analyses (Dantas *et al*., 2010). In healthy subjects, there may be a good reproducibility of temporal parameters with short (<24 h) or long (>24h) recording periods (Marks and Lightfoot, 1999), but not always in short-term spectral parameters (Lobnig *et al*., 2003). Time domain parameters under relatively stable conditions may be considered reproducible (Hohnloser, 1992), whereas the frequency domain parameters may have a greater degree of dispersion depending on the variability of factors influencing them (Pitzalis *et al*., 1996). However, the application of HRV during a short time period is reported to be more preferred in evaluating changes in HR because external variations have less influence on the HR changes (Malik, 1996; Parati *et al*., 2006). To reduce the influence of external factors, ECG recording is suggested to be performed in stable conditions, including a quiet environment, controlled breathing, and subjects using the same position during different sessions and staying awake as well as being mentally relaxed (Zhong *et al*., 2005; Porges, 2009). RSA mediated by the fluctuation on baroreflex drive on vagal motor neurons during the predominant parasympathetic state (supine position), decreases in the

predominant sympathetic state (upright position) (Saul *et al.*, 1991). Therefore, HRV parameters measured in the supine position which is predominantly controlled by the parasympathetic nervous system are more sensitive to external factors which may disrupt the vagal control (Pizalis *et al.*, 1996). Conversely, the upright position which is predominantly controlled by the sympathetic nervous system is less affected by such external factors and improves the intra-individual stability of HRV indices (Pizalis *et al.*, 1996). Thus, during a situation where external factors a not well controlled, the upright position less affected by external variations is more preferred than the supine position which is more influenced by external factors. The intra-individual measurements can show good reproducibility in the supine position if the external factors are well controlled. In addition, the modification of HRV parameters can be considered physiologically useful only if their measurements are reproducible (Dantas *et al.*, 2010). The supine position is preferred when the aim is to assess the subject in situations with a predominance of cardiac vagal modulation of the HR, and the upright position preferred when the aim is to assess the subject in situations with a predominance of sympathetic activity (Dantas *et al.*, 2010). In short term recordings (5-10 minutes) the external control of respiratory rate increases the HRV reproducibility by reducing the possibility of spontaneous variation; such measurements need to be obtained under very stable conditions in which as many of the influencing factors as possible are kept under control (Pitzalis *et al.*, 1996).

2. 2. Aim of the Experiment

HRV parameters are considered as indexes of autonomic modulation of cardiac activity (Thayer *et al.*, 2012). However, the use of HRV to determine the neural autonomic balance directed at the heart is controversial because the modulation of heart beats is strongly influenced by environmental conditions such as noise capable of disrupting the cardiac autonomic balance (Danatas *et al.*, 2010; Lobnig *et al.*, 2003). We hypothesised that if in our laboratory, external factors capable of affecting the cardiac autonomic balance are not were well controlled, the variations of the HRV parameters could be associated with the environmental conditions, rather than being only the results of ingested stimuli in young healthy subjects laying in supine position. Therefore, the objective of this study was to investigate reproducibility and responsiveness of HRV parameters and QTc interval when assessing cardiac autonomic efferent activity of a typical experiment over the time course in our laboratory and whether the habituation effect associated with a second exposure to the protocol procedures would alter these parameters.

3. 3. Methods and Materials

Seven healthy volunteer subjects (mean age: 23.31 ± 4.01 years) took part in two experimental sessions evaluating the effects of time on the reproducibility of HRV and QTc interval parameters and the influence the habituation during a second exposure to the protocol procedures when assessing cardiac autonomic efferent activities. Subjects were fitted with equipment for cardiac monitoring (ECG), ventilation assessment (respiratory belt transducer), and blood pressure measurement (sphygmomanometer). After 20 minutes of acclimatisation,

alternative periods of 5 minutes recording sessions and 15 minutes rest were performed over 2 hours to investigate the time dependent effects on the cardiovascular autonomic regulation. 7 days later, a second visit was organised to investigate the effects of habituation to the full experiment protocol in these volunteers. HRV parameters and QTc intervals were reported as changes from their respective mean baseline values and all the requirements as outlined in the chapter 2 were observed. Point zero indicates the baseline, followed by different recording sessions according to various intervals. Reproducibility was assessed using multiple comparisons between-groups and within-groups (ANOVA with Bonferroni post hoc testing) to evaluate changes between the mean values of each time point to every 5 minutes recorded during the two visits. The habituation to the protocol at the second exposure was evaluated by comparing equal time points between the two visits (paired t-test).

3. 4. Results

Subjects remained normotensive over the time course (Kshirsagar *et al.*, 2006). The time spent on a therapy table in the semi-supine position during the recording period did not have any effect on BP with subjects' SBP and DBP remaining statistically unchanged from their respective baseline values ($p>0.05$) during the two visits (table 3). In addition, their respective mean values at each equal time point are statistically the same between the two visits.

Time (min)	BP (mmHg) at first visit		BP (mmHg) at second visit	
Period recorded (min)	SBP	DBP	SB	DBP
0-5	128 ± 3	77 ± 1	129 ± 2	77 ± 3
20-25	128 ± 3	78 ± 2	129 ± 2	79 ± 2
40-45	130 ± 2	80 ± 2	128 ± 3	81 ± 3
60-65	127 ± 2	78 ± 2	129 ± 3	80 ± 3
80-85	130 ± 4	82 ± 2	129 ± 3	79 ± 3
100-105	127 ± 3	74 ± 2	139 ± 3	76 ± 2
120-125	126 ± 3	80 ± 2	125 ± 4	79 ± 2

Table 3: SBP (mmHg) and DBP (mmHg) mean values (± SEM) show no significant differences over time from respective mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$).

During the time course of the protocol, subjects responded with no significant changes ($p>0.05$) in mean values (± SEM) of HR (bpm) over the time course from mean baseline values at both the first (mean 1.72 ± 2.21 beats/min decrease, $p>0.05$) and the second (mean 1.49 ± 3.75 beats /min reduction, $p>0.05$) visits, compared with their respective mean baseline values (table 4 and figure 7). Each equal time point first versus second visits shows also no significant differences in mean HR ($p>0.05$), and multiple comparisons between-groups and within-groups show unchanged HR ($p>0.05$) at each time point compared with every 5 minute recorded during the two visits.

Time (min)	HR (bpm) at first visit	HR (bpm) at second visit
0-5	63.91 ± 2.62	66.07 ± 4.37
20-25	62.25 ± 2.60	64.57 ± 3.76
40-45	61.53 ± 1.90	63.57 ± 4.23
60-65	61.19 ± 1.51	62.91 ± 3.22
80-85	60.75 ± 2.45	63.85 ± 3.65
100-105	62.67 ± 2.10	65.72 ± 3.80
120-125	63.07 ± 2.26	65.33 ± 3.22

Table 4: HR (bpm) mean values (± SEM) indicate no significant differences over the time course from mean baseline values at both visits ($p>0.05$).

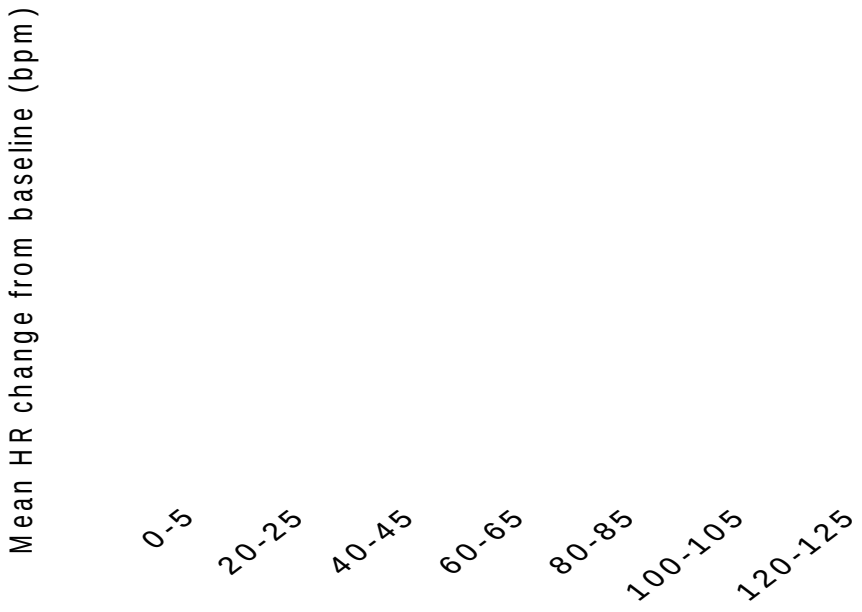

Figure 7: Change from mean (± SEM) baseline HR (bpm) during the first and second visits over the time course. No significant differences over the time course from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups do not show any statistical changes at both visits ($p>0.05$).

In addition, mean values (± SEM) of cardiac interval RMSSD (msec) indicate no significant differences over the time course from mean baseline values ($p>0.05$) during the first (mean 1.77 ± 14.46 msec increase, $p>0.05$) and second (mean 1.33 ± 10.50 msec increase, $p>0.05$) visits from their respective baseline values (table 5 and figure 8). There are also no significant changes in mean cardiac interval RMSSD (msec) at each equal time point first versus second visits. Multiple comparisons between-groups and within-groups show no significant differences in cardiac interval RMSSD over the time course ($p>0.05$).

Time (min)	RMSSD (msec) at first visit	RMSSD (msec) at second visit
0-5	55.54 ± 12.50	54.63 ± 10.75
20-25	61.94 ± 16.36	59.83 ± 8.39
40-45	61.14 ± 15.41	56.76 ± 10.25
60-65	61.22 ± 14.81	59.34 ± 11.61
80-85	55.57 ± 13.98	57.24 ± 12.42
100-105	55.88 ± 13.62	50.69 ± 10.40
120-125	49.92 ± 12.49	53.17 ± 9.70

Table 5: Cardiac interval RMSSD (msec) mean values (± SEM) indicate no significant differences over the time course from mean baseline values at both visits ($p>0.05$).

Figure 8: Change from mean (± SEM) baseline cardiac interval RMSSD (msec) during the first and second visits over the time course. No statistically significant differences over time from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups do not show any statistical changes at both visits ($p>0.05$).

Furthermore, mean PNN50 (%) does not show any significant differences at either the first (mean 1.9 ± 8.33 % increase, $p>0.05$) or the second (mean 1.08 ± 7.98 % increase, $p>0.05$) visits, compared with their respective baseline values (table 6 and figure 9). There are also statistically unchanged mean cardiac HF power at both the first (mean 6.98 ± 5.08 nu decrease, $p>0.05$) and the second (mean 3.60 ± 5.19 nu decrease, $p>0.05$) visits from their respective baseline values (table 7 and figure 10). Each equal time point first versus second visits and multiple comparisons between-groups and within-groups

show no significant differences in both PNN50 and HF power over the time course ($p>0.05$).

Time (min)	PNN50 (%) at first visit	PNN50 (%) at second visit
0-5	29.62 ± 8.38	30.06 ± 8.33
20-25	34.16 ± 9.10	33.73 ± 8.50
40-45	33.73 ± 9.34	32.37 ± 8.37
60-65	33.41 ± 8.06	34.43 ± 7.99
80-85	31.39 ± 10.21	31.98 ± 8.37
100-85	31.86 ± 9.28	26.61 ± 7.36
120-125	26.92 ± 9.40	28.83 ± 6.93

Table 6: Cardiac interval PNN50 (%) mean values (± SEM) indicate no significant differences over the time course from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$).

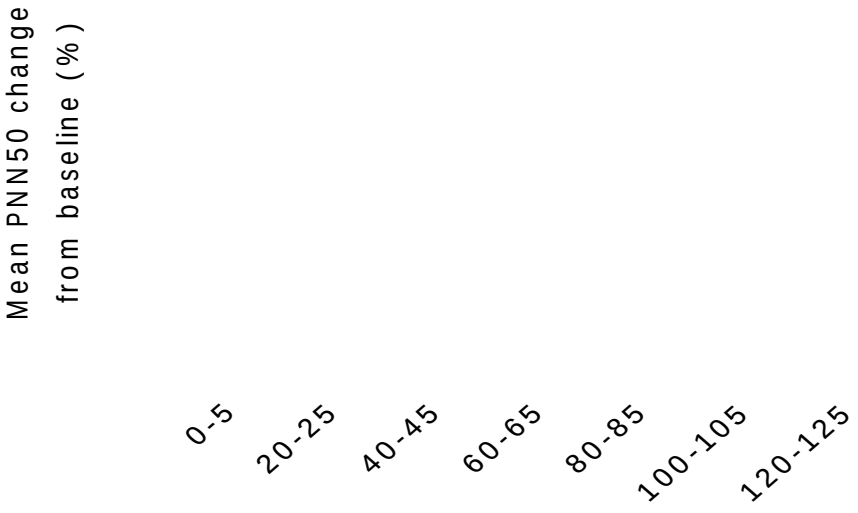

Figure 9: Change from mean (± SEM) baseline cardiac interval PNN50 (%) during the first and second visits over the time course. No significant differences over time from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups do not show any statistical changes at both visits ($p>0.05$).

Time (min)	HF (nu) at first visit	HF (nu) at second visit
0-5	71.79 ± 6.62	71.87 ± 4.71
20-25	68.60 ± 6.17	70.26 ± 6.53
40-45	67.70 ± 3.76	67.48 ± 5.69
60-65	67.95 ± 2.95	68.39 ± 4.35
80-85	67.91 ± 2.91	65.87 ± 5.29
100-105	69.19 ± 3.35	70.84 ± 3.40
120-125	67.44 ± 3.91	70.57 ± 5.30

Table 7: Cardiac interval HF power (nu) mean values (± SEM) indicate no significant differences over the time course from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$).

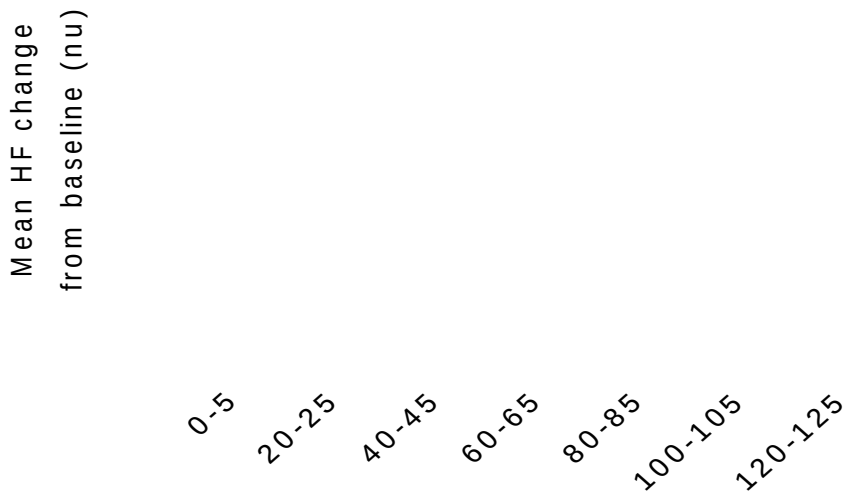

Figure 10: Change from mean (± SEM) baseline cardiac interval HF power (nu) during the first and second visits over the time course. No significant differences over time from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups with HF do not show any statistical changes at both visits ($p>0.05$).

Moreover, subjects responded with no significant effects in mean QTc interval over the time course from mean baseline values during the first (mean 2.69 ± 8.04 msec decrease, $p>0.05$) and second (mean 2.24 ± 11.03 msec decrease, $p>0.05$) visits, compared with their respective baseline values (table 8 and figure 11), as well as at each time point between the two visits ($p>0.05$). Likewise, multiple comparisons between-groups and within-groups indicate no significant differences in QTc interval ($p>0.05$) during the experimental protocol of the two visits.

Time (min)	QTc interval (msec) at first visit	QTc interval at second visit
0-5	416.37 ± 8.17	413.86 ± 11.70
20-25	411.55 ± 9.51	410.02 ± 10.72
40-45	410.06 ± 8.39	409.41 ± 11.45
60-65	413.28 ± 9.04	412.33 ± 10.34
80-85	411.11 ± 7.77	413.25 ± 10.82
100-105	413.58 ± 6.30	413.85 ± 11.56
120-125	413.97 ± 7.40	410.82 ± 11.01

Table 8: QTc interval (msec) mean values (± SEM) show no significant differences over the time course from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$).

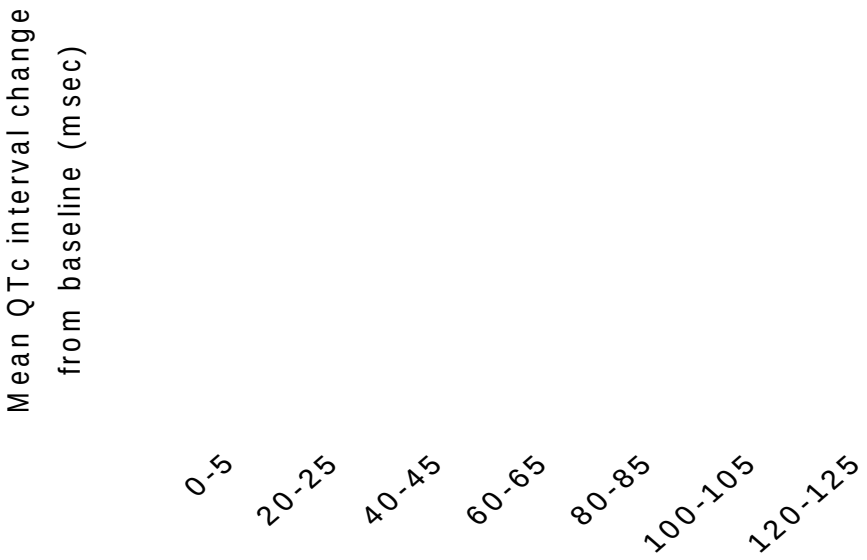

Figure 11: Change from mean (± SEM) baseline QTc interval (msec) during the first and second visits over the time course. No significant differences over time from mean baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups do not show any statistical changes at both visits ($p>0.05$).

Finally, there are no significant changes in LF power (nu) during the first (mean 3.07 ± 5.14 nu increase, $p>0.05$) and the second (mean 2.00 ± 5.25 nu increase, $p>0.05$) visits (table 9 and figure 12) and no significant differences at either the first (mean 0.08 ±0.15 ratio increase, $p>0.05$) or second (mean 0.05 ± 0.11 ratio increase, $p>0.05$) visits in mean LF/HF ratio (table 10 and figure 13), from their respective mean baseline values over the time course. The comparison of each equal time points first versus second visits shows no significant changes in both LF (nu) and LF/HF ratio between the two visits, and all multiple comparisons between groups and within groups with both LF power

and LF/HF ratio do not show any statistical changes at both visits ($p>0.05$) during the experimental protocol.

Time (min)	LF (nu) at first visit	LF (nu) at second visit
0-5	25.25 ± 7.08	24.50 ± 5.32
20-25	29.64 ± 6.41	22.42 ± 4.95
40-45	92.46 ± 4.59	30.96 ± 6.32
60-65	28.95 ± 3.68	26.75 ± 5.29
80-85	29.39 ± 3.85	27.18 ± 4.84
100-105	26.56 ± 3.95	25.98 ± 4.32
120-125	30.53 ± 5.10	27.75 ± 5.71

Table 9: Cardiac interval LF power (nu) mean values (± SEM) indicate no significant differences over the time course from mean baseline values at both visits, and no significant changes at any equal time points first versus second visits ($p>0.05$).

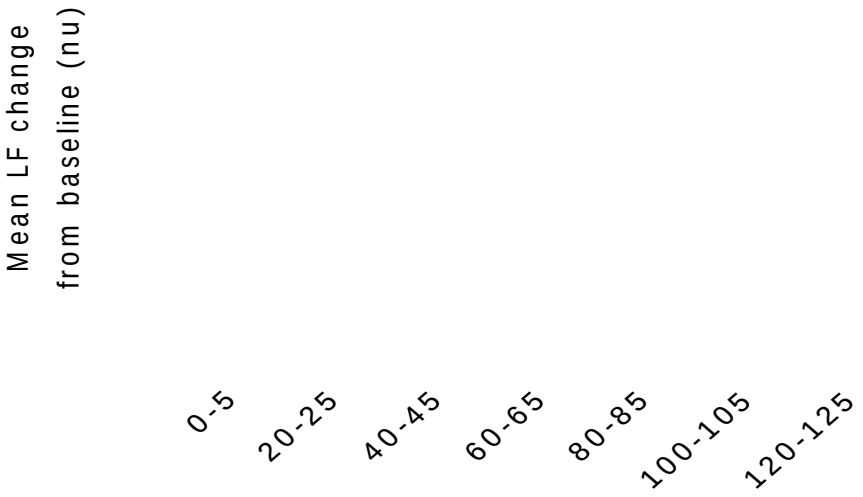

Figure 12: Change from mean (± SEM) baseline cardiac interval LF power (nu) during the first and second visits over the time course. No significant differences over time from mean baseline values at both visits ($p>0.05$) and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups do not show any statistical changes at both visits ($p>0.05$).

Time (min)	LF/HF ratio at first visit	LF/HF ratio at second visit
0-5	0.45 ± 0.19	0.38 ± 0.10
20-25	0.64 ± 0.25	0.36 ± 0.05
40-45	0.61 ± 0.19	0.65 ± 0.19
60-65	0.45 ± 0.07	0.43 ± 0.10
80-85	0.66 ± 0.16	0.52 ± 0.13
100-105	0.40 ± 0.07	0.37 ± 0.07
120-125	0.55 ± 0.10	0.40 ± 0.11

Table 10: LF/HF (ratio) mean values (± SEM) indicate no significant differences over the time course from baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups with LF/HF ratio do not show any statistical changes at both visits ($p>0.05$).

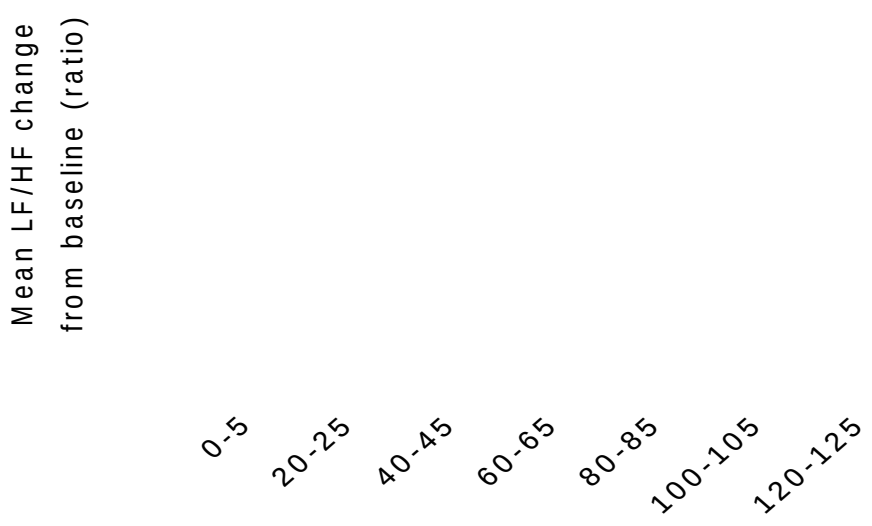

Figure 13: Change from mean (± SEM) baseline cardiac interval LF/HF ratio during the first and second visits over the time course. No significant differences over time from baseline values at both visits and no significant changes at any equal time points first versus second visits ($p>0.05$). Multiple comparisons between groups and within groups do not show any statistical changes at both visits ($p>0.05$).

3. 5. Discussion

Although some studies have shown good reproducibility in HRV analysis (Marks and Lightfoot 1999), and other have reported the opposite (Lord *et al.*, 2001), the current study showed no cardiovascular changes over the time course, indicating reproducibility of data in short-term spectral and temporal analyses as well as in brief QTc interval evaluation. In addition unchanged HRV parameters and QTc interval values at each equal time points between the two visits over the time course, suggests that all values were not influenced by the second exposure to the protocol procedures. Analyses of the frequency domain supplying information concerning the overall variance in HR from periodic

oscillations at various frequencies (Pitzalis *et al.*, 1996), the reproducibility of these results indicates that the measurements were made under relatively stable conditions. The time domain analyses evaluated by means of individual 5-min recordings under our relatively stable conditions show reproducibility of data, confirming observations from previous studies (van Hoogenhuyze *et al.*, 1991; Pitzalis *et al.*, 1996). The autonomic dysfunction with a contribution from sympathetic and parasympathetic branches causes cardiac effects by over activity of either the sympathetic nervous system by various factors, including mental stress (sin *et al.*, 2010) or the parasympathetic tone (Samuels, 2007), leading to HR variations. The results observed in this experiment showing no variations in the parasympathetic activity as indicated by unchanged cardiac interval RMSSD, indicate that the time spent during the recording period did not alter the cardiac vagal tone. In addition, unchanged sympathetic tone seen in this study as shown by no change in cardiac QTc interval (Taubel *et al.*, 2012) , suggests that our recordings were obtained under stable conditions which reduce factors that might activate the sympathetic activity which may impair reproducibility, including local conditions (noise), mental stress, paced breathing, and position (Dantas *et al.*, 2010). Besides the unchanged sympathetic tone monitored with QTc interval analysis, the unaffected sympatho-vagal activity (LF power) also indicates that the activities of the two branches of cardiovascular autonomic regulation were unaffected over the time course. Intra-individual variability can also occur due to the intrinsic instability of HRV parameters consequent to changes of mood, alertness, and mental activity, although these parameters are difficult to control (Dantas *et al.*, 2010).

To reduce the influence of these factors, ECG were obtained in a silent room and subjects stayed awake and mentally relaxed throughout the recording periods. The analysis of HR provides information on neural and non-neural regulatory activity and in this study, LF/HF ratio providing a measure of flexibility of the sympatho-vagal balance was reproducible, indicating no change in the sympatheto-vagal balance and confirming that the modulating factors were minimized. The supine position, more sensitive to variations of the sympathetic activity caused by external factors such as stress and sleep (Reland *et al.*, 2005), is more preferred when the aim is to assess the subjects in situations with a predominance of cardiac vagal modulation of the HR (Dantas *et al.*, 2010). The results from this experiment indicate that intra-individual measurements can show good reproducibility for short-time recordings in the supine position in resting subjects as far as external factors capable of altering autonomic balance are well controlled. Studies questioning reproducibility of HRV parameters evaluated during different times of day indicated some significant differences when measurements were carried out at different times; morning (08:00h-09:00h), earlier afternoon (12:00h-13:00h), and late afternoon (15:00h-16:00h) in supine position (Lord *et al.*, 2001). Situations in which the same experiment should be obtained at different times in the same individual, the reproducibility of data was obtained only when the ECG was recorded in the upright position (Dantas *et al.*, 2010). Contrarily, the results of this study showed good reproducibility of both time and frequency parameters of HRV despite the semi-supine position probably due to the fact that all recordings were pereformed during morning time which minimised the

experimental impact of the variation in the baseline autonomic function known to occur over the course of the day (Akerstedt and Gillberg, 1983). In these subjects, the time dependent effects on and the second exposure to the protocol procedures did not have any effects on cardiac interval RMSSD, cardiac interval PNN50, cardiac HF power, all indicating unchanged cardiac vagal tone and on QTc interval indicating unchanged sympathetic activity, therefore providing mechanisms to explain unchanged HR (Task Force, 1996). The unchanged HRV parameters and QTc interval values at each equal time points first versus second visits indicate that the second exposure to the protocol procedures did not influence the autonomic cardio-vascular activity.

In conclusion, there were no time-dependent variations on cardiac autonomic efferent activity over the time course and there was no habituation effect of the second exposure to the protocol procedures in these healthy human volunteers at rest. Thus, by keeping the same well controlled environement and the same position, the time dependent effects on either HRV parameters or QTc interval and the reproducibility of data, when quantifying cardiac parasympathetic and sympathetic autonomic efferent activity in our young healthy volunteers do not evoke any cardiovascular autonomic changes. The application of HRV during a short period (5 min) with well controlled external factors can be preferred in determining cardiovascular changes in this semi-supine position (Pitzalis *et al.*, 1996; Task Force, 1996; Malik, 1996; Parati *et al.*, 2006). Therefore, different cardiac autonomic efferent activity variations in further investgations will be associated with the effects of ingested stimulus rather than being related to the

time spent during the recording period or to the second exposure to the protocol procedures.

CHAPTER 4: THE EFFECTS OF GASTRIC DISTENSION AND TEMPERATURE ON CARDIAC AUTONOMIC EFFERENT ACTIVITY IN HEALTHY HUMAN VOLUNTEERS

4. 1. Introduction

Gastric stretch is known to induce an increase in muscle sympathetic nerve activity and BP (gastrovascular reflex), to increase HR and to generate an elevation in coronary vasoconstriction without primarily causing significant changes in the left ventricular inotropic state in healthy people (Rossi *et al*., 1998; Van Orshoven *et al*., 2004), probably associated with the redistribution of cardiac output. A burden placed on the CVS due to a distended stomach by food, increases blood flow in the superior mesenteric artery, maybe due to the release of vasoactive intestinal hormones, and to chemical properties of food which decrease splanchnic arterial resistance and allow an elevation in blood flow in order to supply adequate oxygen to the GI tract and support an effective absorption of nutrients (Seth *et al*., 2008). The vasodilation reduces both TPR and BP, but in the normal situation systolic BP does not fall after a meal in young healthy subjects due to the gastrovascular reflex (Van Orhoven *et al*., 2004). The physiological mechanism of this reflex is considered to be a direct neural effect of stomach distension mediated by stretch receptors in the stomach wall via vagal afferent fibres (Rossi *et al*. 1998). The systemic BP is maintained due to compensation by baroreflex mechanisms which increase both CO and peripheral arterial resistance in order to prevent a fall in systemic BP during and after the meal (Gallavan *et al*., 1980; Fujimura *et al*., 1997). The increase in the systemic resistance and the splanchnic vasodilatation are the

main determinants which allow the redistribution of blood flow where it is needed (Gallavan *et al.*, 1980). The gastric afferent limb during gastric distension is mediated by the vagus nerve which sends signals to the NTS, whereas the efferent limb involves the sympathetic pathway which operates via splanchnic nerve fibres, perhaps combined to the activation of renin-angiotensin system (Molinary *et al.*, 2003). Vagal GI neurons are sensitive to mechanical stimulus and express a variety of mechanical sensitive TRP ion channels, and TRPV4, TRPP, TRPA1 receptors are prime candidate stretch- sensitive ion channels (Huang, 2004; Nilius and Owsianik, 2011). However, when gastric distension becomes accompanied by pain, the TRPV1 gastric nociceptive receptors are activated and convey via splanchnic afferent fibres, information to the central nervous system (Grundy and Scratchered, 2011). The presence of gastric TRPM8 thermoreceptors known to be activated by temperatures below 28°C (Story *et al.*, 2003, Fajardo *et al.*, 2008), suggests that when the food is ingested at the temperature below usual room temperatures (21-22°C) (Burdon *et al.*, 2012), the cold temperature may interact with the stretch induced increased HR and BP, which might result in a different cardiovascular response.

4. 1. 1. Transduction Mechanisms during Gastric Stretch

The ENS contains the reflex pathways that control secretion, motility and blood flow associated with digestion and absorption (Blackshaw *et al.*, 2007) and does not have any projections outside the gut wall to the CNS and therefore no obvious afferent role (Bertrand and Thomas, 2004). However, the extrinsic ANS which includes the sympathetic and parasympathetic nervous systems, influences effector function by modulating enteric reflexes and conveys sensory

information which provides a basis for spinal and brainstem reflex mechanisms that regulates digestive function, inputs to central autonomic circuits that regulate feeding, and gives rise to both painful and non-painful sensations (Blackshaw *et al.*, 2007). Vagal (prevalent in the proximal gut) and spinal (predominate in the distal gut) gastrointestinal sensory afferent neurons project centrally to the brainstem and spinal cord with their peripheral projections ending at various levels within the gut wall including the muscle, mucosal epithelia, serosa, mesenteric attachments and enteric ganglia (Blackshaw *et al.*, 2007). The transduction mechanisms involved in vagal afferent sensors seem to be similar to gustative receptors (salt, sour, bitter, sweet, and umami) which when stimulated, act via specific ion channels or receptors, including Na^+ ion channel, H^+ ion channel, G protein- coupled receptor (GPCR), and induce intracellular signalling cascades, which ultimately generate the release of neurotransmitters which activate the vagal afferent nerve (Berthoud *et al.*, 2000). This similarity has led to the cells in the gut being referred to as intestinal taste cells (Blackshaw *et al.*, 2007). These cells have an apical tuft of microvilli exposed to the intestinal lumen with the ability of monitoring luminal contents and in response to an appropriate stimulus, release the neurotransmitters such as the 5-HT across the basolateral membrane to activate afferent terminals in close proximity within the lamina propria (Blackshaw *et al.*, 2007). The afferent vagal response to gastric stretch consists of three major components during and immediately after distension: A dynamic response consisting of an initial burst with high frequency discharge due to an initial high tension developed during an active resistance offered by smooth

muscle and local excitatory reflexes produced by intrinsic nerves, followed by a static or sustained response, and finally by a pause or silent period after the end of the distension (Blackshaw *et al*. 1987). The central target of vagal afferent nerve fibres is mainly the NTS (Kubin *et al*., 2006). The information can be relayed to other parts of the medulla, the hypothalamus (depressor area and vasopressin-producing, magnocellular neurons of supraoptic and paraventricular nuclei) and the cerebellum (Mack *et al*., 2001). Neuroimaging studies indicate that gastric stretch also activates the midbrain (where most dopamine neurons are located), the pons (where serotonergic nuclei are located), hypothalamus (involved in the control of food intake), amygdala (limbic region implicated in emotional reactions to food), and thalamus (implicated in arousal) (Tomasi *et al*., 2009). Within the medulla, the output from NTS follows the baroreflex pathways already described in the general introduction. Nevertheless, under some circumstances, abdominal sensation related to gut stimulus can be consciously perceived, indicating that afferent pathways can have projections to the nuclei in both the medulla, and the brain cortex which includes the primary somatosensory cortex of the thalamus responsible for physical sensation (Matthews and Aziz, 2005). The consciously perceived sensations include satiety, nausea, bloating, discomfort and pain (Shea *et al*., 2000). Mechanosensitive afferent neurons responding to gastric stretch are classified in low- and high-threshold mechanosensitive fibres (Shea *et al*., 2000). Low threshold neurons are activated by regulatory functions, including storage, propulsion and emptying (Ozaki and Gebhart, 2001). The same neurons are also stimulated by stimuli leading to conscious sensations associated with non-

painful mechanical stimulation, such as the sensation of fullness, bloating and nausea (Cerve and Janig, 1992). Contrarily, high threshold responses connected to nociceptors give rise to discomfort and acute pain which is conveyed to the central nervous system by gastric splanchnic afferent fibres (Grundy and Scratchered, 2011) when the distension pressure is equal to or exceed 30 mmHg (Ozaki *et al*., 2001). During prolonged gastric stretch, adapting mechanoreceptors in the mucosa are stimulated, combined to other factors such as muscle/nerve fatigue or enzyme exhaustion (Seth *et al*., 2008), which allow different parameters such as BP to return to baseline.

4. 2. Aim of the Experiment

Gastric distension is reported to increase both the HR and BP, little is known about the influence of gastric cooling combined with gastric stretch. We hypothesized that when gastric stretch is combined with gastric cooling, the activation of gastric TRPM8 cold receptors may send vagally mediated temperature-related inputs to the NTS of the midbrain (Fajardo *et al*., 2008), with some connections to the high centres, including the hypothalamus (Cervero *et al*., 1994), probably resulting in the inhibition of the stretch induced HR and BP. Therefore, the aim of this study was to compare the cardiovascular responses to the same volume of gastric distension only and gastric stretch combined to gastric cooling.

4. 3. Methods and Materials

Nine healthy volunteer subjects (mean age 24.08 ±3.12 years) took part in two different experimental sessions according to a randomized crossover study (see experimental protocol). After being fitted with the equipment for cardiac (ECG), ventilation (Pneumotrace II, UFI, Australia), and BP (sphygmomanometer or finger plethysmograph) monitoring, subjects were asked to ingest 300 mL of Fybogel solution, a drink that contains Ispaghula husk, a natural fibre which can help to ease the digestive flow (Saha, 2014). This solution is designed to be a drink that is only slowly absorbed by the stomach (McIntyre *et al*., 1997), so will have either a small and/or a slow effect on ECF volume and concentration in order to distend the stomach. This volume was designed to be a volume which distends the stomach without activating the gastric nociceptive receptors estimated to 30 mmHg associated with bigger elongation of the gastric wall (Grundy and Scratchered, 2011; Vanis *et al*., 2012). Fybogel solution was ingested either at 37°C (isothermic solution) or at 6°C (cold solution) in order to activate either gastric stretch receptors only, or gastric stretch receptors combined with gastric cold receptors respectively. The sphygmomanometer was firstly used to monitor BP at the start and the end of every session because of the lack of a device which could monitor a continuous BP such as finger plethysmograph in our laboratory. After a 5 minute baseline recording, a continuous 20 minute recording session was followed by alternative periods of 5 minutes rest and 5 minutes recording sessions during the first hour, also followed by alternative periods of 10 minutes rest and 5 minutes recording sessions during the second hour as described in chapter 2. However, no BP

could be measured during the first continuous 20 minutes ECG recording to avoid interrupting the continuous ECG recording. Thus, further experiments were carried out whereby the nine subjects were assessed to record changes up to 20 minutes after Fybogel ingestion, but continuously monitored with a finger plethysmograph (courtesy of Dr Michael J. White, University of Birmingham). The procedure and recommendations as described in chapter 2 were observed. In accordance with the general methods and materials, values of HR, cardiac interval RMSSD, QTc interval, and SBP were reported as changes from the average of respective baseline values. Statistical analyses were carried out using one-way ANOVA with Bonferroni post hoc testing to compare each time point over the post-drink period with the respective baseline value and paired-test to compare equal time points of the two visits.

4. 4. Results

Each subject ingested Fybogel solution at the two different temperatures without problem, and none reported any sensation of discomfort, bloating, nausea or pain. No urge to void the bladder was expressed by any of the subjects during the recording period. The resting baseline values of HR, RMSSD, and SBP showed no statistical differences ($p>0.05$) in the experimental groups compared with controls (tables 11-13). Subjects responded to gastric stretch only with a trend towards increased HR (mean 1.20 ± 3.012 beats/ min increase, $p>0.05$) lasting 20 minutes from mean baseline value, whereas cold Fybogel induced a significant decrease in HR (mean 1.81 ± 1.84 beats/min decrease, $p<0.05$) which lasted for 15 minutes with a peak around 10 minutes (mean 2.22 ± 1.79 beats/min decrease, $p<0.01$) (figure 14).

Time (min)	HR (bpm) with gastric stretch effect only	HR (bpm) with gastric stretch and cold effects
-5-0	67.73 ± 2.37	67.85 ± 1.82
0-5	69.67 ± 2.82	67.53 ± 2.12
5-10	69.64 ± 2.37	65.91 ± 1.77*
10-15	68.63 ± 2.72	65.63 ± 1.85**
15-20	67.81 ± 3.02	65.33 ± 1.99*
25-30	65.73 ± 2.66	66.94 ± 1.85
35-35	65.90 ± 2.54	66.57 ± 3.62
45-50	65.56 ± 2.41	66.22 ± 2.00
60-65	66.37 ± 2.56	67.60 ± 1.86
75-80	66.85 ± 2.93	67.70 ± 2.53
90-95	67.08 ± 3.06	67.26 ± 2.72
105-110	66.44 ± 2.69	67.81 ± 2.51

Table 11: HR (bpm) mean values (± SEM) show significant differences (*$p<0.05$, **$p<0.01$) over time from baseline value after Fybogel solution ingested at 6˚C. No significant changes over time from baseline value with Fybogel solution ingested at 37˚C and no significant changes at any time point cold versus isothermic Fybogel solution ($p>0.05$).

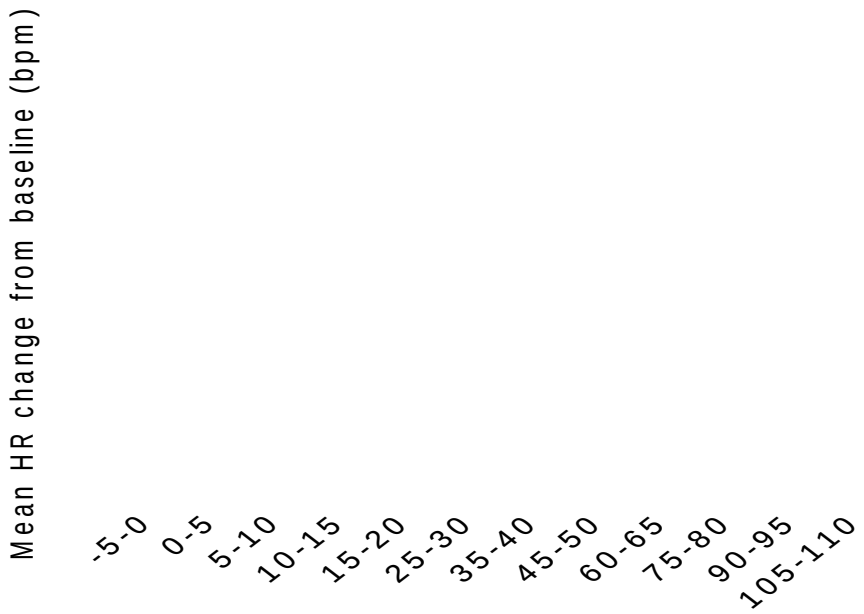

Figure 14: Change from mean baseline HR (bpm) between Fybogel solution ingestion at either body temperature or cold visits (± SEM) over the time course. *p<0.05, **p<0.01, significant differences over time from baseline value with cold Fybogel solution. No significant differences over time from baseline value with isothermic Fybogel solution and no significant changes at any time point cold versus isothermic Fybogel solution (p>0.05).

The assessment the index of cardiac vagal tone with Fybogel at body temperature indicates no significant differences in mean cardiac interval RMSSD (mean 7.87 ± 8.12 msec increase, p>0.05) from mean baseline value over the time course (figure 15). However, when the stomach was distended with cold Fybogel, the results show a significant increase in mean cardiac interval RMSSD between 5 and 20 minutes compared with mean baseline (mean 13.93 ± 16.12 msec increase, p<0.05)

Time (min)	RMSSD (msec) with gastric stretch effect only	RMSSD (msec) with gastric stretch and cold effects
-5-0	70.64 ± 11.05	73.36 ± 12.61
0-5	74.04 ± 12.52	79.66 ± 14.80
5-10	72.05 ± 14.01	87.76 ± 14.64*
10-15	76.29 ± 16.24	91.59 ± 15.01**
15-20	74.48 ± 16.56	87.97 ± 14.52**
25-30	75.48 ± 16.65	86.91 ± 14.74
35-40	76.09 ± 16.54	86.79 ± 15.79
45-50	77.27 ± 15.62	86.12 ± 16.54
60-65	75.07 ± 14.01	75.55 ± 12.44
75-80	79.20 ± 16.76	76.72 ± 17.75
90-95	76.17± 16.61	74.45 ± 15.41
105-110	78.24 ± 15.93	73.75 ± 15.68

Table 12: Cardiac interval RMSSD (msec) mean values (± SEM) indicate significant differences (*$p<0.05$, **$p<0.01$) over time from baseline value after Fybogel solution ingested at 6°C. No significant changes ($p>0.05$) over time from baseline value with Fybogel solution ingested at 37°C and no significant changes at any time point cold versus isothermic Fybogel solution ($p>0.05$).

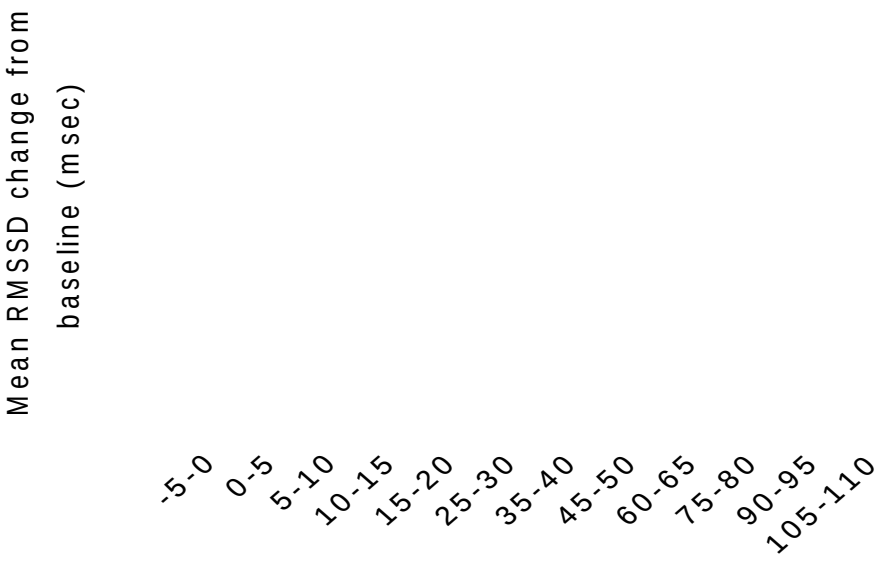

Figure 15: Change from mean baseline cardiac interval RMSSD (msec) with Fybogel solution ingestion at either body temperature or cold visits (± SEM) over the time course. *p<0.05, **p<0.01, significant differences over time from baseline value with cold Fybogel solution. No significant differences over time from baseline value with isothermic Fybogel solution and no significant changes at any time point cold versus isothermic Fybogel solution (p>0.05).

However, the monitoring of the index of cardiac sympathetic activity after ingestion of Fybogel at body temperature indicates an immediate significant decrease in mean QTc interval (mean 12.65 ± 4.98 msec decrease, p<0.05) which lasted for 20 minutes compared with mean baseline value (figure 16). Nevertheless, cold Fybogel drinking indicates no significant changes in QTc interval (p>0.05).

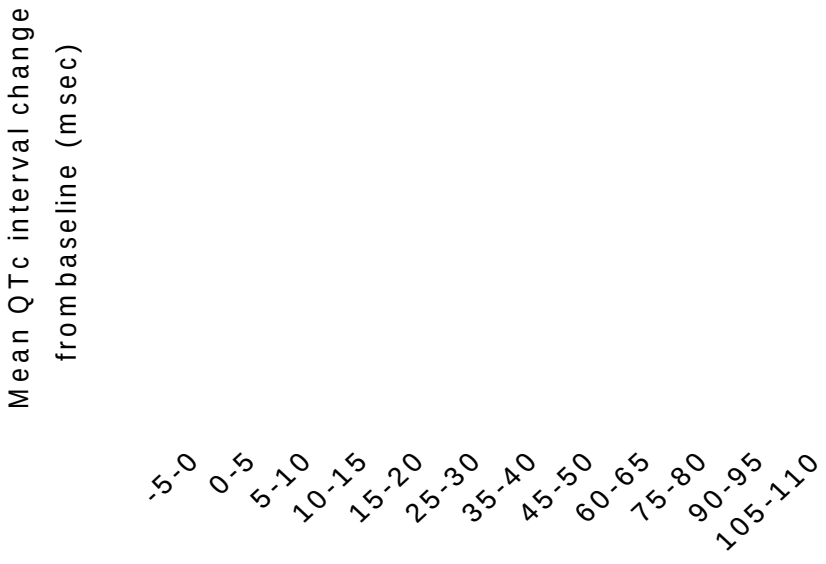

Figure 16: Change from mean baseline cardiac QTc interval (msec) with Fybogel solution ingestion at either body temperature or cold visits (± SEM) over time. *p<0.05, **p<0.01, significant differences over time from baseline value with isothermic Fybogel solution. No significant differences over time from baseline value with cold isothermic Fybogel solution (p>0.05).

The evaluation of the BP response to gastric stretch, show that subjects responded to Fybogel at body temperature with a slight increased mean SBP (mean 2.26 ± 2.36 mmHg increase, p>0.05) from mean baseline, with a peak around 5 minutes before its starts to decrease toward the baseline level (figure 17). However, cold effect appears to induce a trend towards decreased SBP after cold Fybogel ingestion (mean 0.84 ± 2.37 mmHg decrease, p>0.05) over time, despite the initial 5 minutes slight increase (mean 0.78 ± 2.22 mmHg increase, p>0.05) from mean baseline value.

Time (min)	SBP (mmHg) with gastric stretch effect only	SBP (mmHg) with stretch and cold effects
-5-0	118 ± 3	118 ± 3
0-5	123 ± 3	118 ± 2
5-10	Not recorded	Not recorded
10-15	Not recorded	Not recorded
15-20	Not recorded	Not recorded
25-30	120 ± 3	117 ± 3
35-40	121 ± 3	114 ± 3
45-50	121 ± 3	116 ± 2
60-65	118 ± 3	113 ± 2
75-80	121 ± 3	119 ± 2
90-95	119 ± 3	115 ± 2
105-110	120 ± 2	118 ± 3

Table 13: SBP (mm Hg) mean values (± SEM) indicate no significant differences over time ($p<0.05$) from baseline value with Fybogel solution ingested at either 37˚C or 6˚C. No blood pressure was recorded between 5 and 20 minutes using sphygmomanometer.

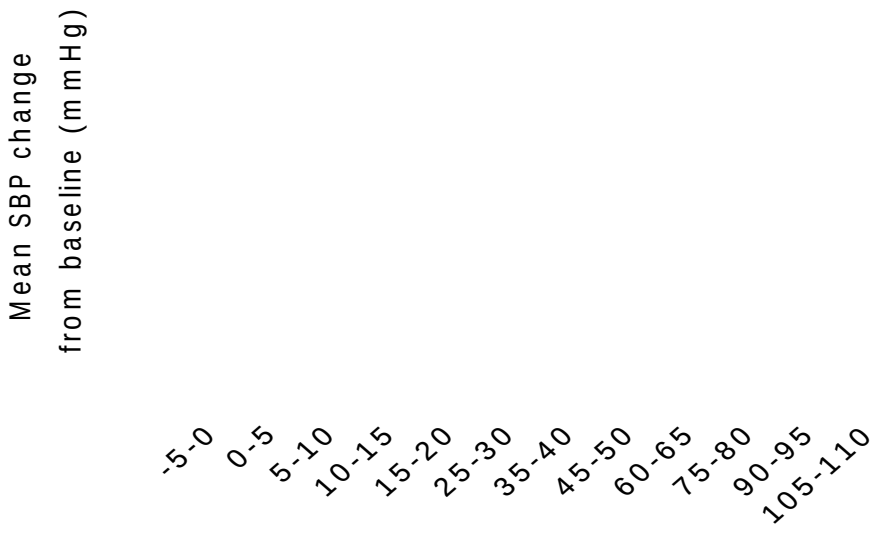

Figure 17: Change from mean baseline SBP (mmHg) with Fybogel solution at either body temperature or cold visits (± SEM) over the time course. No significant differences over time from baseline value with Fybogel solution at either body temperature or cold temperature.

Nevertheless, no BP was measured using the sphygmomanometer during the first 20 minutes immediately subsequent to Fybogel ingestion. Thus, a 20 minute short-onset recording session using a finger plethysmograph device (table 14), shows a general trend towards increased SBP over time (mean 3.83 ± 4.47 mmHg increase, $p>0.05$) compared with mean baseline with Fybogel at body temperature, with a peak during the first 5 minutes (figure 18). Like in the 2 h experiment session, subjects responded to gastric stretch and cold stimuli with a trend towards decreased SBP (mean 0.19 ± 4.59 mmHg decrease, $p>0.05$) between 5 to 20 minutes from mean baseline value. There are significant changes ($p>0.05$) in both DBP and MAP with Fybogel ingestion at both temperatures (tables 15 and 16).

Time (min)	SBP (mmHg) with gastric stretch effect only	SBP (mmHg) with stretch and cold effects
-5-0	115 ± 5	118 ± 2
0-5	120 ± 4	119 ± 3
5-10	119 ± 5	117 ± 2
10-15	118 ± 5	118 ± 3
15-20	118 ± 5	117 ± 2

Table 14: SBP (mmHg) mean values (± SEM) show no significant differences ($p > 0.05$) over time from baseline value with Fybogel solution ingested at both temperatures during the short-onset recording session.

Time (min)	DBP (mmHg) with gastric stretch effect only	DBP (mmHg) with stretch and cold effects
-5-0	59 ± 3	59 ± 3
0-5	61 ± 3	60 ± 4
5-10	62 ± 3	59 ± 3
10-15	63 ± 3	60 ± 4
15-20	63 ± 4	60 ± 4

Table 15: DBP (mmHg) mean values (± SEM) show no significant differences ($p > 0.05$) over time from baseline value with Fybogel solution ingested at both temperatures during the short-onset recording session.

Time (min)	MAP (mmHg) with gastric stretch effect only	MAP (mmHg) with stretch and cold effects
-5-0	78 ± 3	79 ± 3
0-5	81 ± 3	80 ± 4
5-10	81 ± 3	78 ± 3
10-15	81 ± 2	79 ± 4
15-20	81 ± 3	79 ± 3

Table 16: MAP (mmHg) mean values (± SEM) show no significant differences ($p > 0.05$) over time from baseline value with Fybogel solution ingested at both temperatures during the short-onset recording session.

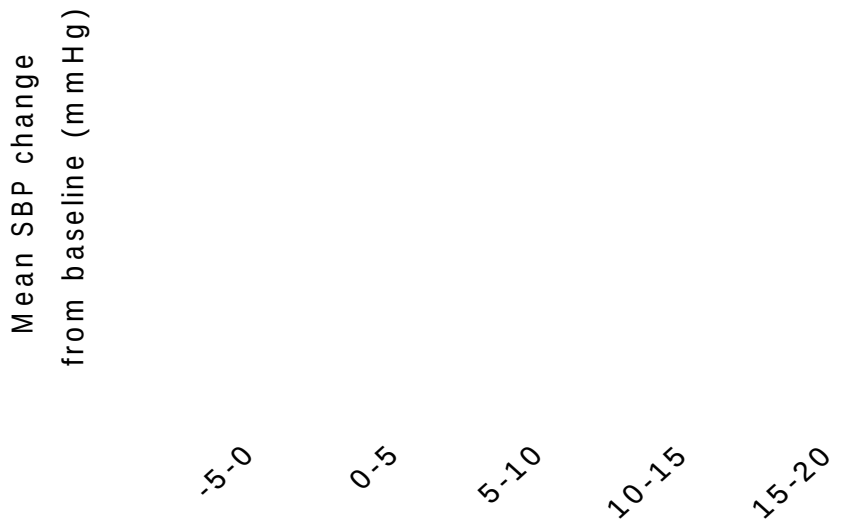

Figure 18: Change from mean baseline SBP (mmHg) with Fybogel solution ingestion at either body temperature or cold visits (± SEM) during the short-onset recording session. Fybogel ingestion at body temperature shows a trend towards increased SBP, whereas cold Fybogel indicates a general trend towards decreased SBP.

The reassessment of QTc interval response during the short-onset also shows a significant decrease in mean QTc interval with gastric stretch only (mean 13.60 ± 5.02 msec decrease, $p<0.05$) over the 20 minutes period, whereas gastric stretch and cold stimuli shows a decrease of decreased QTc interval (4.81± 5.29 msec decrease, $p>0.05$), compared with the QTc interval with gastric stretch only over the time course (table 17 and figure 19).

Time (min)	QTc interval (msec) with gastric stretch only	QTc interval with gastric stretch and cold effects
-5-0	375.08 ± 5.47	374.99 ± 4.45
0-5	360.90 ± 5.19**	370.43 ± 5.28
5-10	360.28 ± 5.47**	370.44 ± 4.45
10-15	359.71 ± 4.23**	369.17 ± 5.22
15-20	365.04 ± 5.85*	370.68 ± 5.43

Table 17: QTc interval (msec) mean values (± SEM) indicate significant differences (**p<0.01, ***p<0.001) over time from baseline value with isothermic Fybogel solution during the short-onset recording session. No significant differences (p>0.05) over time from baseline value with cold Fybogel solution.

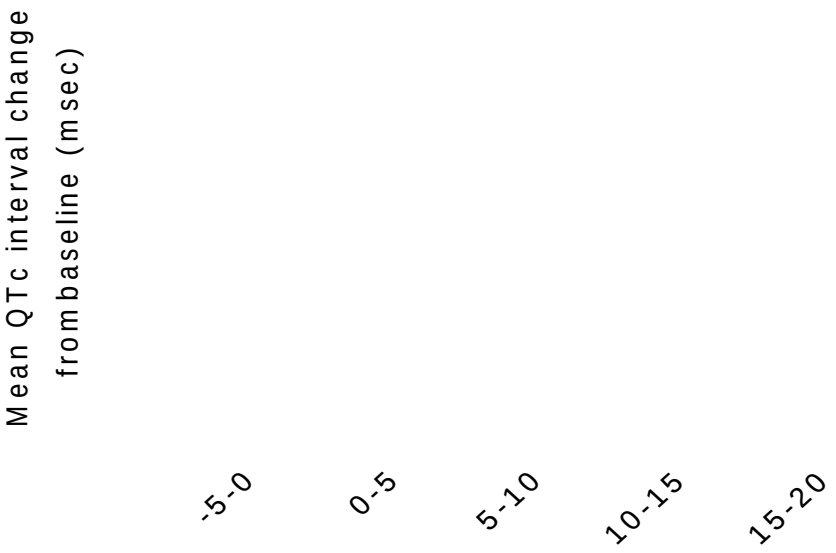

Figure 19: Change from mean baseline cardiac QTc interval (msec) with Fybogel solution ingestion at either body temperature or cold visits (± SEM) during the short-onset session. *p<0.05, **p<0.01, significant differences over time from baseline value with isothermic Fybogel solution. No significant differences over time from baseline value with cold Fybogel solution

4. 5. Discussion

The main finding of this study is that gastric distension achieved by 300 mL of isothermic Fybogel ingestion showed a slight increase in BP, probably due to an increase in the sympathetic nervous system indicated by QTc shortening. The shortening in QTc interval may be explained by a prolonged duration in sympathetic activation (30sec-5 min) associated with higher concentration in noradrenaline in ventricular muscles and Purkinje neurons known to decrease the cardiac action potential and induces a sympathetically mediated shortening in QT interval (Apkon and Nerbonne,1988). However, gastric stretch only appeared having no effect on cardiac vagal activity as shown by unchanged cardiac interval RMSSD.

These results are consistent with findings from another observation where gastric distension was achieved by a balloon inflated to a volume of 300 ml, induced a sympathetic enhancement which prevented the fall in SBP due to an intraduodenal glucose infusion (3 kcal/min) in healthy older subjects (Vanis *et al.*, 2012). The results of our study are also in line with another study where gastric distension to a volume of 700 mL generated an increase in both the muscle sympathetic nerve activity and MAP from 92 ± 3.8 mmHg to 99 ± 3.7 mmHg (Van Orshoven *et al.*, 2004). Cardiac sympathetic stimulation is regarded as a direct neural effect of gastric stretch mediated by tension receptors in the stomach wall which send afferent inputs to the CNS via vagal afferent nerves to increase BP as explain in the introduction. The sympathetic activation obtained in this experiment lasted only for 20 minutes probably due to the cessation of gastric stretch when Fybogel solution started to enter the duodenum as it is known to help easing the digestive flow (Saha, 2014). The investigation of the rate of gastric emptying of a radiolabelled isosmotic drink of orange juice indicated that the half time (t½) for emptying of the drink ingested at 4°C was 18.7 (2.6) min, whereas the drink ingested at 37°C was 14.0 (1.7) min (Sun *et al.*, 1988). Gastric distension plays a protective role in the regulation of postprandial blood pressure and may be interpreted as an effect which counteracts the fall in systemic BP due increased superior mesenteric artery blood flow associated with decreased splanchnic arterial resistance (Seth *et al.*, 2008). The BP is reported to increase more in the elderly people where the decrease in splanchnic arterial resistance induced by gastric distension is less pronounced than in young subjects as ageing causes reduction in central

arterial compliance with the reduced ability to show vasodilatation (Monahan *et al*. 2001). In healthy sedentary subjects, arterial compliance is progressively reduced by 40–50% between the ages of approximately 25 and 75 years (Tanaka *et al*., 2001). The decline in artery compliance with age may explain why the BP did not increase too much in our young healthy subjects. However, the study evaluating the effects of gastric distension with a balloon at volumes of 100, 300, and 500 mL on BP indicated that gastric distension at all volumes prevented the decrease in BP expected to occur with the intraduodenal glucose infusion in healthy older people and the magnitude of this attenuation was associated with the intragastric distension volume (Vanis *et al*., 2012). This suggests that the second reason why the BP did not increase that much may be related to the small gastric distension because the increase in BP after gastric distension is directly proportional to the volume of the distension (Seth *et al*., 2008). This finding may explain a result from another observation where gastric distension obtained by the inflation of a bag placed in the stomach with 400 mL of air induced a significant increase in SBP probably due to larger elongation of gastric wall and stronger activation of stretch receptors (Vanis *et al*., 2010). The increase in SBP after gastric stretch may be related to stimulation of TRPV4, TRPP, and maybe TRPA1 (Huang, 2004; nilius and Owsianik, 2011) reported to send afferent stretch-related information to the NTS via vagal fibre nerves (Molinary *et. al*., 2003). In return, the NTS sends efferent sympathetic inputs via splanchnic nerve fibres to induce arterial vasoconstriction via the activation of α_1-adrenergic arterial receptors (Longhurst and Ibarra, 1982) which may prevent a drop in systemic BP due increased splanchnic blood flow (Seth *et al*.,

2008). In addition, cardiac sympathetic activation mediated by $ß_1$- adrenergic receptors increases HR, pumping force, and conducting velocity (Stavrakis *et al.*, 2011). The inhibitory baroreflex resetting expected to counteract the sympathetic outflow and causes a decrease in HR (Fadel and Raven, 2011) may have been overridden by the sympathetic activation, leading to only a slight increased HR observed in this study. Besides confirming these results, the study reported here indicates a cold mediated cardiac vagal stimulation indicated by increased cardiac interval RMSSD, supplying a mechanism to explain decreased HR. This cardio-vagal activation may have masked the stretch mediated sympathetic activation (Paton *et al.*, 2005), combined with the corresponding decreased HR, and may have led to a fall in cardiac output (Scott *et al.*, 2001) and to a substantial decrease in the workload to the heart. HR with stretch and cold stimuli decreased only for 20 minutes, and started to increase towards the baseline level probably due the cardiac sympathetic reintroduction due to the cessation of cold effect as cold Fybogel is warmed up quickly in the body to reach intra-abdominal body temperature level. The underlying transduction mechanisms of cold sensitivity being reported in vagal afferent nerve fibres in mammalian cell lines (Story *et al.*, 2003), suggests that changes associated with a stimulation of thermosensitive sensory afferent vagal neurons may be triggered by the stimulation of gastric TRPM8 thermoreceptors (Girona *et al.*, 2014), known to be activated by temperature below 28°C (Farardo *et al.*, 2008). Afferent sensory neurons then convey to the NTS temperature-related information (Girona *et al.*, 2014), which results in a decline in HR probably via direct inhibitory inputs to the cardiac SA through the NA (Paton et al., 2005) or

indirectly via the CVLM pathways (Bailey *et al.*, 2006) by activating cardiac muscarinic M_2 receptors (Stavrakis *et al.*, 2011). The cold mediated sympathetic inhibition may occur in the NTS where the two limbs meet during the central pathway as explained in the general introduction.

In general, this study and others (Van Orshoven *et al.*, 2004; Vanis *et al.*, 2012) gave persuasive evidence that gastric distension induces an increase in sympathetic nervous system activity, leading to an increase in BP in young subjects. This direct neural cardiac sympathetic activity, causing an increase in BP (both SBP and DBP), HR and CO, maybe speculated to be an anticipatory mechanism that plays a protective role in the maintenance of postprandial BP. However, when gastric distension is combined with gastric cold stimulus, the decrease in HR and BP reflex responses may be mediated by both sympathetic withdrawal and increased parasympathetic activity. The inhibition of sympathetic activation, the corresponding decrease in HR, and the substantial decrease in workload to the heart may be a pertinent observation in clinical use when patients with cardiovascular pathologies distend the stomach by cold food, although further studies are needed to address the role of stomach distension combined with gastric cooling in human pathologies.

CHAPTER 5: EFFECTS OF PEPPERMINT OIL INGESTION ON CARDIAC AUTONOMICEFFERENT ACTIVITY IN HEALTHY HUMAN VOLUNTEERS

5. 1. Introduction

Peppermint oil (PO) derives from the Mentha piperita plant, readily extracted by steam distillation and its main content is menthol (Papathanasopoulos *et al.*, 2013) among other volatile oils, including menthone, limonene, cineole, menthofuran, isomenthone, methyl acetate, pulegone, and carvone (Tate, 1997). PO is used as flavouring in food and beverage, can be taken orally in dietary supplement (Meamarbashi, 2014), and its consumption safety was proven in toxicological assessments (Nair, 2001). It has been used in indigenous medicine for various therapeutic benefits, including the treatment of irritable bowel syndrome as a spasmolytic agent (Johnson *et al.*, 2009). A study including 175 patients in five trials found a statistically significant benefit of peppermint oil compared with placebo in the symptomatic treatment of irritable bowel syndrome (Kligler and Chaudhary, 2007).Menthol is a cyclic terpene alcohol ($C_{10}H_{20}O$) which activates the TRPM8 receptor (Andersson *et al.*, 2004), a menthol sensitive receptor which also responds to cold temperature ranged between 30–15 °C considered innocuous cool (McCoy *et al.*, 2011). Menthol can also activate TRPV1 receptors, giving an open possibility to express some nociceptive actions conveyed to the CNS via Aδ- and C-fibres (Campero *et al.*, 2001; Green, 2005). Thermosensitive afferent neurons have also been identified projecting to the oesophagus, stomach, duodenum, intestines, respiratory tract, and vasculature (Tsuzuki *et al.*, 2004; Johnson *et al.*, 2009). Ingestion of peppermint enteric-coated capsules releases its menthol content in

the stomach which activates the TRPM8 cold receptors and potentiates synaptic transmission independent of its effects at peripheral nerve endings (Tsuzuki *et al.*, 2004). Afferent sensory neurons of the DRG are the site of detection with select afferent populations activated at distinct temperature thresholds; innocuous cool (30-15˚C) and noxious cold (<15˚C) (McKemy, 2013). The stimulation of TRPM8 converts thermal stimuli into neural activity at the level of the primary afferent nerve (Jordt *et al.*, 2003). Menthol induces spontaneous glutamate release via its actions at presynaptic terminals of the first somatic sensory synapses between primary afferent fibres and the dorsal horn (DH) neurons in the spinal cord or the equivalent brains regions (Tsuzuki *et al.*, 2004). TRPM8 channels are synthesized in the DRG neuron and transferred to the terminals by axonal transport (figure 20). Peripheral TRPM8 receptors activated the menthol, generates action potential that transfers the stimulus information to the central terminal of the DRG neuron, resulting in an enhancement of spontaneous release of l-glutamate onto spinal substantia gelatinosa neurons, which play a pivotal role in modulating cold temperature transmission in a rat (Kumamoto *et al.*, 2014).

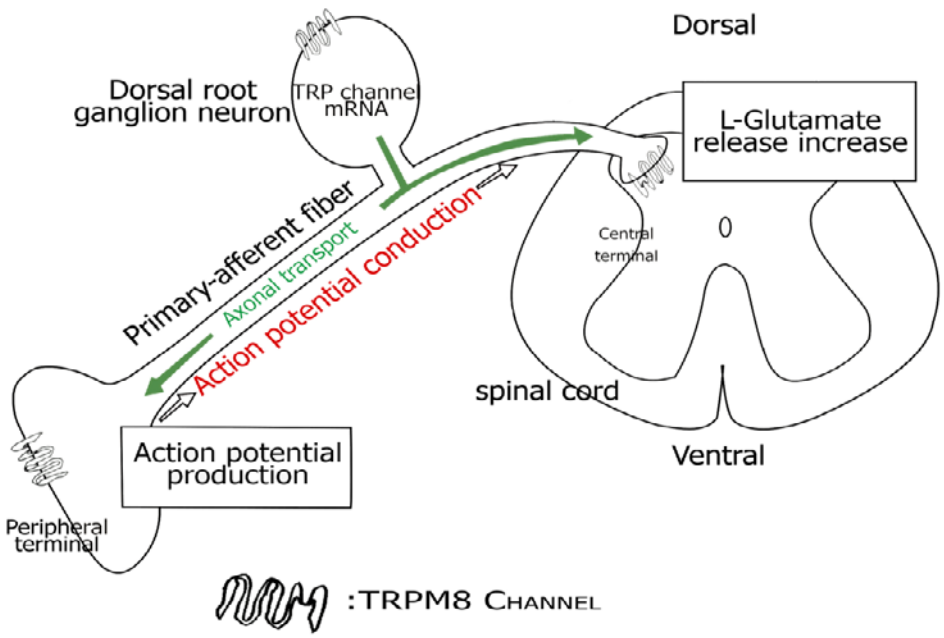

Figure 20: Role of TRPM8 channels in the peripheral and central terminals of DRG neuron in transmitting sensory information. TRPM8 channels are synthesized in the DRG neuron and transferred to the peripheral terminal (Kumamoto *et al.*, 2014).

Spontaneous glutamate release might mostly be induced by the mobilisation of intracellular Ca^{2+} stores at presynaptic terminals, perhaps also by a direct Ca^{2+} entry through menthol-activated cation channels, presumably via the activation of : TRPM8 chnnel ›rs expressed on the plasma membranes of DRG presynaptic terminals (Tsuzuki *et al.*, 2004). The menthol-induced Ca^{2+} release was argued to not be coupled with a conventional intracellular pathway because U73122, the phospholipase C (PLC) inhibitor used to block inositol triphosphate (IP_3) production, did not block menthol-induced increase of the intracellular Ca^{2+} level in a Ca^{2+} free bath solution (Tsuzuki *et al.*, 2004). One possibility is that TRPV1 is also expressed on endoplasmic reticulum, and its

activation can directly mobilize intracellular Ca^{2+} stores (Olah *et al.*, 2001; Liu *et al.*, 2003). However, several laboratories have found that TRPM8 activity is associated with the presence of the membrane lipid phosphatidylinositol 4, 5-biphosphate (PIP_2), the substrate for phospholipase C (PLC) (Rohacs *et al.*, 2005; Johnson *et al.*, 2009). Potassium channels have a role in regulating neural excitability by resetting membrane potential, keeping fast action potentials short, terminating periods of intense activity and modulating the time between successive action potentials (Hille, 1978). However, studies investigating the responses of isolated nodose ganglion neurons to the thermal stimuli in a mouse also indicated an increase in cytoplasmic-free Ca^{2+} concentration in response to cooling below 28°C or to cooling agents such as menthol and icilin (Zhan *et al.*, 2004). These results show that nodose ganglion which contains the nerve cell bodies of vagal afferent neurons that receive afferent innervation from the abdominal and thoracic viscera are also activated by cooling agents, including menthol via the activation TRPM8 receptors as confirmed by immunohistochemistry animal studies (Zhan *et al.*, 2004). Therefore, both vagal and splanchnic fibres appear to be activated by thermal stimuli and cooling agents such as menthol in gut (El Ouazzani and Mei, 1982), but in the oesophagus and stomach which are densely innervated by vagus nerve fibres, it is likely that thermal stimuli uses vagal afferent pathway to transmit thermal inputs the NTS under normal physiological conditions (Zhan *et al.*, 2004; Fajardo *et al.*, 2008). In return NTS conveys vagal efferent inputs to cardiac SA node via NA to decrease HR (Paton *et al.*, 2005).

5. 2. Aim of the Experiment

Peppermint oil is available as a constituent of medicines (Nath *et al*., 2012), but not much is known about it effects on the cardiovascular system in healthy people. However, we hypothesised that the activation of TRPM8 receptors by its menthol content known to enhance cardiac vagal tone may induce various cardiovascular responses, including a decreased HR. Therefore, this experiment aims to assess the cardiovascular autonomic efferent variations in response to menthol after coated capsules of peppermint oil ingestion, without activating the cardiac sympathetic tone observed with gastric stretch and cooling (chapter 4), in young healthy volunteers.

5. 3. Methods and Materials

Nine subjects (mean age: 24.31 ± 3.18 years) gave informed consent to take part in morning sessions where subjects were asked to ingest coated capsules of PO. A preliminary session was undertaken to determine the likely time for ingested coated capsules of peppermint oil to release its menthol content in the stomach. Coated capsules of PO were stirred up in 0.1 M HCl at 37°C (similar to stomach environment) in a Dissolution Apparatus (Varian 705 DS, USA). The time taken for 9 coated capsules of PO to disintegrate was evaluated and mean 8 ± 0.42 minutes was obtained for coated capsules of PO to break up in the 0.1 M HCl. This time could be estimated near to the time taken for coated capsules to release its menthol content in the stomach after ingestion. After being fitted with equipment for cardiac monitoring (ECG), ventilation assessment (pneumotrace respiratory strap) and continuous blood pressure measurement (finger plethysmograph), subjects were asked to swallow 2 coated capsules of

PO (200mg, Boots Pharmaceuticals, UK) using 10 ml of isothermic isotonic saline solution instead of water because water may induce various cardiovascular responses, including increase both vagal and the sympathetic activity (Brown *et al.*, 2005). Coated capsules of PO are commercially available in the UK and are completely tasteless and odourless, and generally recognized as safe (Sohi *et al.*, 2004; Papathanasopoulus *et al.*, 2013). Subjects fully complied with the general materials and methods requirements.

Values of HR (bpm), cardiac interval RMSSD (msec), QTc interval (msec), and SBP (mmHg) were reported as changes from the average of respective baselines. Statistical analyses were carried out using one way Anova with Bonferroni post hoc testing to compare each time point over the post-drink period with the baseline value in order to evaluate the effects of peppermint oil on the CVS.

5. 4. Results

None of the subjects reported any discomfort or nausea after capsules coated of PO ingestion. The ingestion of coated capsules of PO induces a significant decrease in mean HR (mean 3.71 beats/min decrease, $p<0.05$) between 25-50 minutes from mean baseline values (table 18, figure 21).

Time (min)	HR (beats/min)
-5-0	62.22 ± 3.12
0-5	61.71 ±3.21
5-10	62.17 ± 3.30
10-15	61.40 ± 3.22
15-20	61.83 ± 3.66
25-30	57.85 ± 2.41*
35-40	58.06 ± 2.40*
45-50	58.17 ± 2.51*
60-65	60.55 ± 2.64
75-80	60.11 ± 2.39
90-95	60.77 ± 2.15
105-110	62.56 ± 2.59

Table 18: HR (beats/min) mean values (± SEM) indicate significant differences (*$p<0.05$) over time from mean baseline value after coated capsules of PO ingestion.

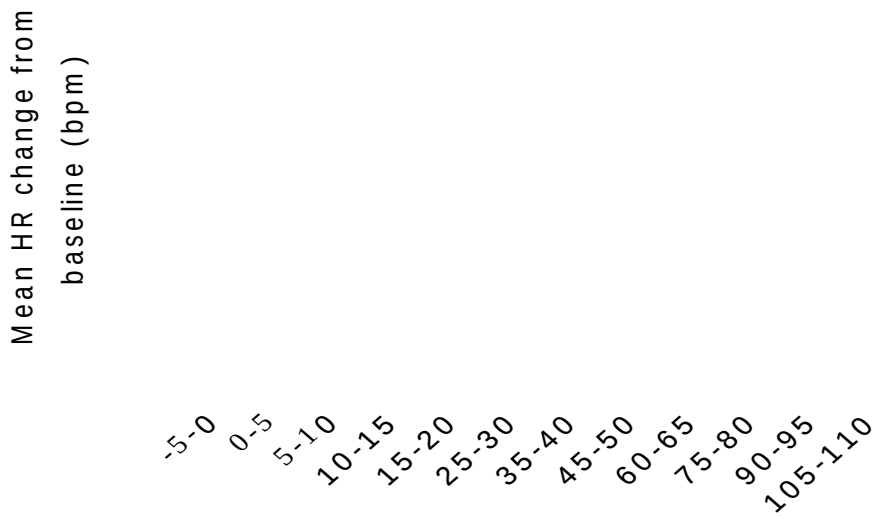

Figure 21: Mean change in HR (beats/min) after coated capsules of PO ingestion (± SEM) over the time course. *p<0.05 significant differences over time from mean baseline value.

The evaluation of cardiac vagal activity shows a significant increase in mean cardiac interval mean cardiac interval RMSSD (mean 19.43 ± 13.55 msec increase, $p<0.05$) between 25-50 minutes from mean baseline value (table 19, figure 22).

Time (min)	RMSSD (msec)
-5-0	110.42 ± 14.99
0-5	113.25 ± 15.10
5-10	112.55 ± 14.63
10-15	111.65 ± 15.07
15-20	122.40 ± 15.17
25-30	132.54 ± 13.45*
35-40	132.22 ± 10.86*
45-50	134.79 ± 10.33*
60-65	122.00 ± 16.92
75-80	115.75 ± 16.78
90-95	113.87 ± 18.58
105-110	111.30 ± 16.28

Table 19: Cardiac interval RMSSD (msec) mean values (± SEM) indicate significant differences (*$p<0.05$) over time from mean baseline value after coated capsules of PO ingestion.

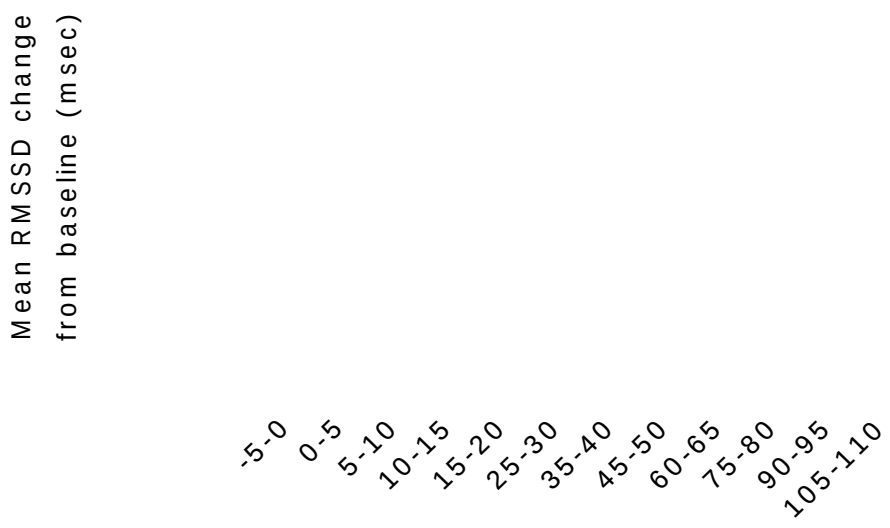

Figure 22: Mean change in cardiac interval RMSSD (msec) after coated capsules of PO ingestion (± SEM) over the time course. *p<0.05 significant differences over time from baseline value.

However, there were no significant effects on mean QTc interval over time from mean baseline value (mean 0.47 ± 6.15 msec increase, $p>0.05$) in response to coated capsules of PO ingestion (table 20 and figure 23).

Time (min)	QTc interval (msec)
-5-0	395.22 ± 7.17
0-5	392.49 ± 8.03
5-10	393.13 ± 6.10
10-15	393.26 ± 6.50
15-20	393.28 ± 6.36
25-30	393.60 ± 5.92
35-40	394.55 ± 5.83
45-50	397.47 ± 5.05
60-65	398.42 ± 5.20
75-80	398.82 ±5.77
90-95	397.94 ± 6.08
105-110	399.90 ± 6.53

Table 20: QTc interval (msec) mean values (± SEM) show no significant changes over time from mean baseline value after coated capsules of PO ingestion.

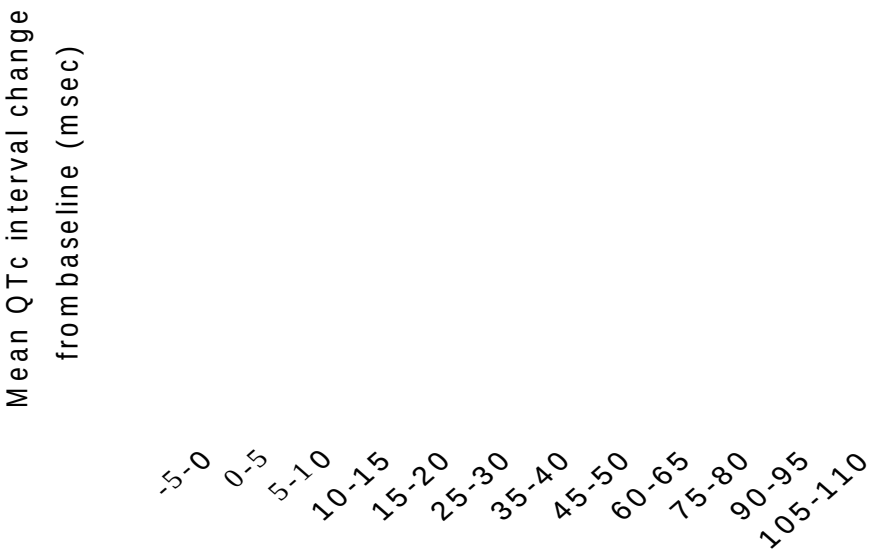

Figure 23: Mean change in QTc interval (msec) after coated capsules of PO ingestion (± SEM) over the time course. No significant changes over the time course from baseline value.

Subjects responded with no significant changes in mean SBP (mean 2.29 ± 4.55 mm Hg increase, $p>0.05$) from mean baseline value in response to coated capsules of PO ingestion over the time course (table 21 and figure 24). However, there is a trend towards decreased SBP between 25 and 45 minutes (mean 1.27 ± 4.38 mmHg decrease, $p>0.05$).

Time (min)	SBP (mmHg)
-5-0	114 ± 5
0-5	115 ± 4
5-10	117 ± 3
10-15	119 ± 4
15-20	119 ± 4
25-30	114 ± 4
35-40	112 ± 5
45-50	113 ± 5
60-65	120 ± 4
75-80	120 ± 6
90-95	117 ± 5
105-110	117 ± 4

Table 21: SBP (mmHg) mean values (± SEM) indicate no significant changes ($p>0.05$) over the time course from mean baseline value after coated capsules of PO ingestion.

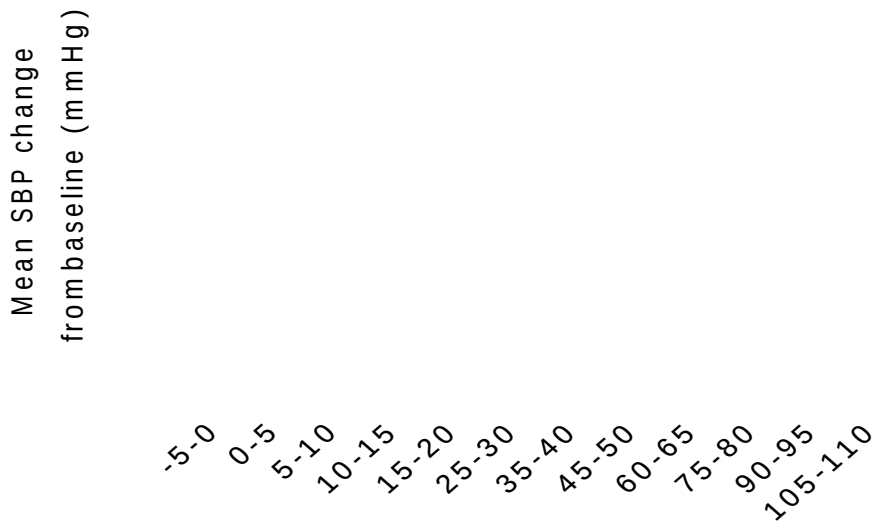

Figure 24: Mean change in SBP (mmHg) after coated capsules of PO ingestion (± SEM) over the time course. There is a trend towards decreased SBP between 25- 45 minutes.

5. 5. Discussion

The main finding of this study was that coated capsules of PO ingestion generated a short-lived increase in cardiac interval RMSSD indicating an increase in cardiac vagal tone with a corresponding reduced HR between 25 and 50 minutes after ingestion, probably related to menthol action on TRPM8 receptors (Baibars *et al.*, 2012). The corresponding slight decrease in SBP may be due to the decrease in vascular resistance (Johnson *et al.*, 2009) as TRPM8 activation reduces arterial smooth muscle tonicity (Meamarbashi, 2014). This effect might explain a not recordable BP observed in a 40 year woman who was brought to an Indian hospital after an ingestion of a toxic dose of oral peppermint oil (Nath *et al.*, 2012), estimated around 1 gram per kilogram of body weight (Baibars *et al.*, 2012). However, time taken between PO ingestion

and the BP fall with the 40 year woman was not estimated as she was brought to the emergency department in a comatosed state. Our results are in line with a finding from another observation assessing the effects of peppermint oil on exercise performance which showed a decrease in HR from 65.18 ± 12.74 to 62.18 ± 11.82 beats/min, $p<0.05$) after ten days of a consumption of 500 mL of water per day containing 0.05 mL peppermint essential oil (Meamarbashi and Rajabi, 2013). Besides confirming these findings, there was no effect on cardiac sympathetic activity, and the effects of menthol on HR reported here were delayed for about 25 minutes, probably due to the time taken for menthol to be release from coated capsules of peppermint oil. Although our preliminary experiment evaluating the time taken for coated capsules of PO to release its menthol content in a solution similar to stomach environment was about 8 minutes, this experiment showed a 25 minute delay for PO to trigger cardiac changes. This inconsistency may be due to the speed at which coated capsules of PO were stirred up in the Dissolution Apparatus which may have not been similar to the speed at which PO moved in the stomach, or to the limited number of subjects which may have not be able to remove individual differences. Another reason may be related to gastric juice secreted from glands lining the stomach containing gastric acid, and other substances such as bile salts and digestive enzymes (Furness *et al.*, 2013) which might have different reactions with coated capsules of PO. The increased cardiac vagal tone without sympathetic involvement and the corresponding reduced HR may induce a decreased workload to the heart. The underlying transduction mechanisms of menthol being reported in afferent sensory neurons in isolated

nodose ganglion, and the presence of a dense vagal innervation in the stomach (Zhan *et al*., 2004), it is possible that changes associated with activation of thermosensitive vagal afferent nerve fibres may be triggered by the stimulation of TRPM8 receptors that convey to the brainstem temperature-related inputs to the NTS (Farardo *et al*., 2008). In return the NTS sends inhibitory inputs to the cardiac SA node to decrease the HR (Bailey *et al*., 2006).

In general, this study showed that beside the reported use of PO in a variety of ailments such as common cold, irritable bowel syndrome, non-ulcer dyspepsia, headache (Nath *et al*., 2012), it may have relevant clinical use when patients with cardiovascular pathologies required cardiac vagal stimulation, a decrease in HR, and a substantial decrease workload to the heart, without any estimated implication of sympathetic activity, although more investigations are needed in human pathologies. Another new finding is that, contrarily to an immediate reduced HR induced by cold Fybogel ingestion (chapter 4), the decrease in HR after PO ingestion has been demonstrated to be delayed for about 25 minutes, probably due to the time taken for PO to release its menthol content in the stomach.

CHAPTER 6: THE EFFECTS OF INGESTION OF ISOTONIC SALINE S OLUTION AND TEMPERATURE ON CARDIAC AUTONOMIC EFFERENT ACTIVITY IN HEALTHY HUMAN VOLUNTEERS

6. 1. Introduction

Water drinking elicits various cardiovascular autonomic responses, including an increase in both total peripheral resistance (TPR) and cardio-vagal activity, and a significant decrease in HR of approximately 4 beats/min ($p<0.01$) in healthy young subjects for about 20 minutes after an ingestion of almost half of a litter of water at 21°C (Brown *et al.*, 2005). The results supported the findings from another observation where the HR fell from 67.6 ± 2.0 (mean ± SEM) to 60.7 ± 2.4 beats/min after 500 mL of water ingestion at 18 °C (Routledge *et al.*, 2002). These cardiovascular changes may be due to gastric distension (Rossi *et al.*, 1998), liquid volume loading effects (Young and Matthias, 2004), or other mechanisms associated with water content, including hypo-osmolality (Shannon *et al.*, 2000; May and Jordan, 2011). Nevertheless, the cardiovascular responses to gastric distension with intragastric bag insufflated with 700 ml air in young subjects were reported to increase muscle sympathetic activity, and HR from 72 ± 4.3 to 76 ± 4.4 beats/min ($p<0.05$) (Van Orshoven *et al.*, 2004), indicating that the mechanisms underlying the cardiovascular responses to water ingestion have additional components to stretch effect. Surprisingly, the ingestion of 517 ± 15 mL of isotonic saline which is expected to generate a greater plasma volume increase than water, failed to elicit the same cardiovascular effects seen with the same quantity of water in young healthy subjects, including no changes in either HR (almost 0.5 beats/min decrease)

with the saline solution, compared with almost 4 beats/min significant reduction in HR ($p<0.01$) with water drinking, 20 minutes after the ingestion (Brown *et al.*, 2005). However, neither water nor saline significantly increased blood pressure (Brown *et al.*, 2005), indicating that the cardiovascular responses to water drinking are not related to volume loading effects, but may be due to water hypo-osmolality (McHugh *et al.*, 2010). Indeed, water hypo-osmolality activates both the sympathetic nervous system and vagal fibres which may be responsible for the cardiovascular changes (Brown *et al.*, 2005), and may have an effect on LF power and LF/HF ratio used as indexes to assess the activity of the two branches of the ANS and the balance between sympathetic and parasympathetic tones respectively. The presence of TRPM8 receptors sensitive to innocuous cold in vagal afferent neurons (Zhao *et al.*, 2009; Lippoldt *et al.*, 2013), suggests that the stimulation of vagal nerves by cooling may send afferent inputs to the NTS which may in turn generate different cardiovascular effects, including changes in HR and BP.

6. 2. Aim of the Experiment

Drinking isotonic saline is reported not to have significant cardiovascular and metabolic effects (Brown *et al.*, 2005), however, there is a little knowledge concerning the influence of cold saline ingestion on the cardiovascular system. Nevertheless, the presence of TRPM8 cold receptors in the stomach (Mustafa and Oriowo, 2005) suggests that cold saline ingestion may activate these receptors and disrupt the cardiovascular sympathovagal balance. Therefore, the aim of this study was to investigate whether the ingestion cold saline may

generate cardiovascular changes and to compare these changes to the results from the same volume of saline solution ingested at the body temperature.

6. 3. Methods and Materials

In accordance with the general methods and materials, nine healthy volunteer subjects (mean age: 23.30 ± 3.15 years) took part in two different sessions assessing the cardiovascular responses to saline solution ingestion. One of the subjects was rejected because he failed to synchronise the breathing the timer operating at 0.2 Hz according to the experimental protocol during several recording periods over the time course. After a 5-min control period, subjects ingested 300 mL of isotonic saline solution (0.9% w/v) at either 37°C or 6°C. The isotonic saline solution was made up using NaCl and mineral water (at both temperatures). ECG, respiratory movements (pneumotrace respiratory strap), and continuous BP (finger plethysmograph) recording sessions were made at predetermined intervals as described in chapter 2. Data were stored anonymously for confidentiality reasons and Offline analysis was carried out from the results of the two visits to compare the cardiovascular responses after isotonic saline ingestion at these two different temperatures. Values of HR (bpm), cardiac interval RMSSD (msec), QTc interval (msec), SBP (mmHg), LF power, and LF/HF ratio were reported as changes from the average of respective mean baseline values. Statistical analyses were carried out using one-way ANOVA with Bonferroni post hoc testing to compare each time point over the post-drink period with the respective baseline value and paired-test to compare equal time points of the two visits.

6. 4. Results

No sensation of discomfort, bloating, nausea or pain was reported by any of the subjects. No urge to void the bladder was expressed by any of the subjects during the recording period. Resting baseline of HR (bpm), RMSSD (msec), QTc interval (msec), LF power (nu), LF/HF ratio and SBP (mmHg) were not statistically different in the experimental group compared with control (tables 22-27).

During the time course of the protocol procedures, subjects responded with a significant decrease in mean HR (mean 4.30 ± 1.57 beats/min decrease, $p<0.05$), between 5 and 30 minutes from mean baseline value in response to cold saline ingestion (figure 24). However, isothermic saline drinking showed no significant decrease in mean HR (mean 1.37 ± 2.48 beats/min decrease, $p>0.05$) over the time course. There is a trend towards increased HR during the first 5 minutes (mean 0.99 ± 2.43 beats/min increase, $p>0.05$) after saline ingestion at the body temperature.

Time (min)	HR (bpm) with isothermic saline solution (37 ºC)	HR (bpm) with cold saline (6 ºC)
-5-0	59.84 ± 2.17	61.43 ± 1.92
0-5	60.83 ± 2.43	59.88 ± 1.91
5-10	59.26 ± 2.53	57.68 ± 1.73*
10-15	58.51 ± 2.35	56.95 ± 1.59**
15-20	58.19 ± 2.38	57.03 ± 1.86*
25-30	57.59 ± 2.23	56.41 ± 1.65*
35-40	57.10 ± 2.68	57.60 ± 1.22
45-50	57.63 ± 2.66	56.96 ± 1.36
60—65	58.73 ± 2.50	56.69 ± 1.73
75-80	58.49 ± 2.43	56.27 ± 1.87
90-95	58.15 ± 2.82	57.08 ± 1.90
105-110	58.50 ± 2.45	57.09± 1.97

Table 22: HR (bpm) mean values (± SEM) show significant differences (*$p<0.05$, **$p<0.01$) between 5 and 30 minutes from mean baseline value with cold saline. No statistical changes ($p>0.05$) over the time course from mean baseline value with isothermic saline ingestion and no statistical differences at any time point cold versus body temperature saline solution.

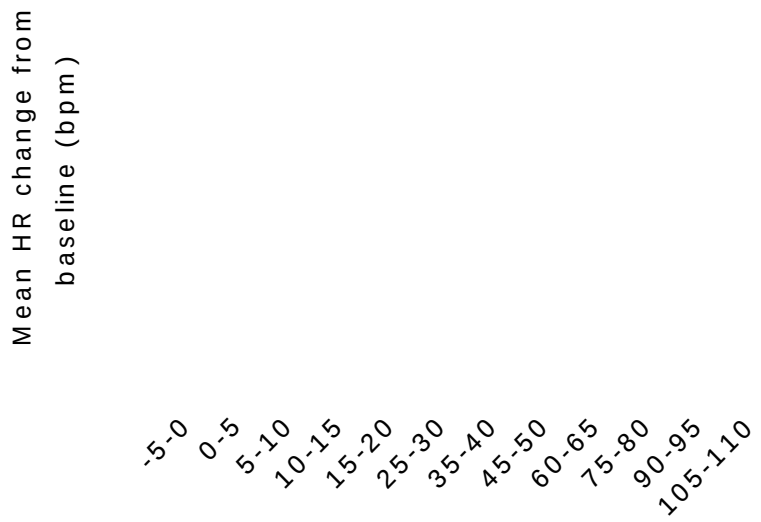

Figure 25: Mean change in HR (bpm) between saline at either body temperature or cold visits (± SEM) over the time course. *p< 0.05, **p<0.01, significant differences between 5 and 30 minutes from mean baseline value with cold saline. No significant changes at any time point cold versus body temperature saline solution.

The evaluation of cardiac interval RMSSD after cold stimulus shows a significant increase in mean cardiac interval RMSSD (mean 29.65 ± 25.17 msec increase, $p<0.05$) between 5 and 30 minutes from mean baseline value (figure 25). Contrarily, isothermic saline ingestion generates no significant differences in mean cardiac interval RMSSD (mean 6.08 ± 20.25 increase, $p>0.05$) over the time course from mean baseline value.

Time (min)	RMSSD (msec) with saline at body temperature	RMSSD (msec) with saline at cold temperature
-5-0	110.76 ± 21.35	100.69 ± 22.54
0-5	119.67 ± 22.10	116.94 ± 21.09
5-10	120.71 ± 27.85	128.56 ± 26.25*
10-15	118.38 ± 24.24	131.83 ± 26.89**
15-20	113.33 ± 19.43	128.26 ± 25.01*
25-30	119.79 ± 20.15	132.71 ± 22.54*
35-40	119.85 ± 19.81	126.32 ± 24.17
45-50	119.82 ± 18.70	118.54 ± 22.45
60-65	113.22 ± 15.42	109.58 ± 22.84
75-80	112.14 ± 17.13	116.94 ± 21.09
90-95	117.55 ± 18.75	112.72 ± 25.06
105-110	113.18 ± 18.58	114.71 ± 24.21

Table 23: RMSSD (msec) mean values (± SEM) indicate significant differences (*$p<0.05$, *$p<0.01$) between 5 and 30 minutes from mean baseline value with cold saline solution. No statistical differences ($p>0.05$) over the time course from mean baseline value with saline ingestion at body temperature and no significant changes at any time point cold versus body temperature saline solution.

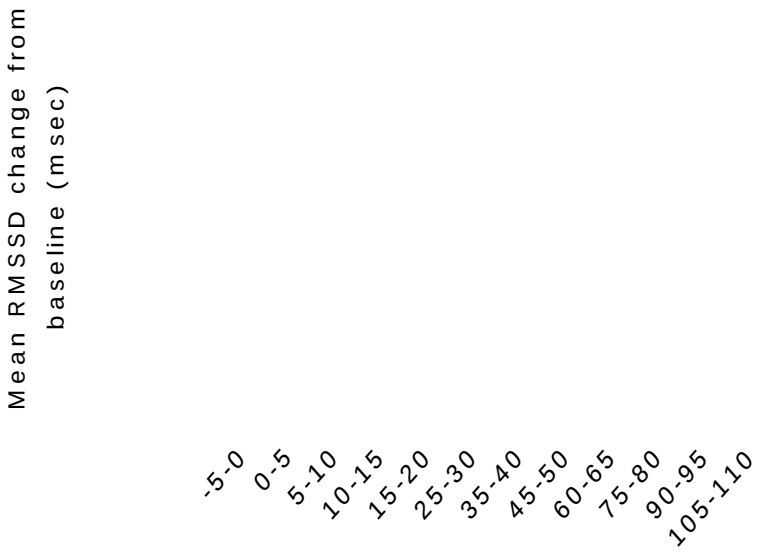

Figure 26: Mean change in cardiac interval RMSSD variability (msec) between saline at either body temperature or cold visits (± SEM) over the time course. *p<0.05, **p<0.01, significant differences between 5 and 30 minutes from mean baseline value with cold saline solution. No significant changes over the time course from mean baseline value with saline ingestion at body temperature and no significant differences at any time point cold versus body temperature saline solution.

The assessment of cardiac sympathetic activity with cold saline ingestion shows a significant decrease in mean QTc interval (mean 8.11 ± 5.39 msec decrease, p<0.05) between 5 and 15 minutes, whereas saline solution at body temperature causes no significant differences in mean QTc interval

(mean 2.18 ± 7.65 msec decrease, p>0.05) over the time course from respective mean baseline values (figure 26).

Time (min)	QTc interval (msec) with saline at body temperature	QTc interval (msec) with cold saline at cold temperature
-5-0	383.94 ± 7.08	385.97 ± 5.82
0-5	380.84 ± 6.70	380.84 ± 5.30
5-10	380.73 ± 6.61	377.89 ± 5.68*
10-15	380.70 ± 7.53	377.82 ± 5.10*
15-20	382.71 ± 7.73	380.80 ± 4.90
25-30	380.09 ± 8.45	379.66 ± 4.80
35-40	381.51 ± 9.25	379.82 ± 5.19
45-50	382.47 ± 7.90	380.49 ± 4.52
60-65	382.38 ± 7.47	379.65 ± 4.97
75-80	381.97 ± 7.75	380.78 ± 5.09
90-95	381.68 ± 8.33	381.67 ± 5.67
105-110	382.22 ± 7.15	384.13 ± 5.45

Table 24: QTc interval (msec) mean values (± SEM) show significant differences (*$p<0.05$) between 5 and 15 minutes from mean baseline value with cold saline solution. No significant differences ($p>0.05$) over the time course from mean baseline value with isothermic saline ingestion and no statistical changes at any time point cold versus body temperature saline solution.

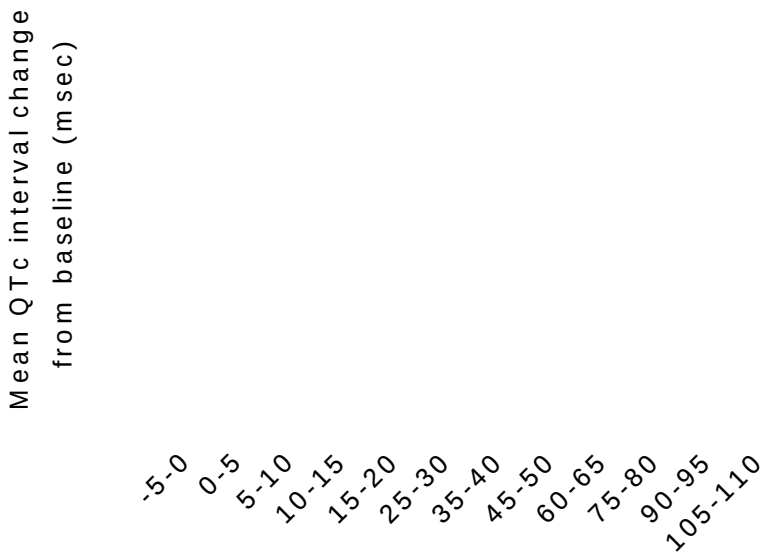

Figure 27: Mean change in QTc interval (msec) between saline at either body temperature or cold visits (± SEM) over the time course. *p< 0.05 significant differences between 5 and 15 minutes from mean baseline value with cold saline solution. No significant differences at any time point cold versus body temperature saline solution.

Over the time course of this protocol, subjects responded with no statistical changes (p>0.05) in mean SBP (mmHg) from mean baseline values with saline ingestion at either cold or body temperatures (figure 27). Nevertheless, there is an initial 15 minute trend towards reduced mean SBP compared with mean baseline value (mean 3.52 ± 3.14 mmHg decrease, p>0.05) with cold saline solution.

Time (min)	SBP (mmHg) with saline at body temperature	SBP (mmHg) with saline at cold temperature
-5-0	122 ± 3	120 ± 7
0-5	124 ± 4	116 ± 3
5-10	124 ± 4	116 ± 3
10-15	124 ± 4	118 ± 4
15-20	124 ± 4	121 ± 4
25-30	124 ± 4	123 ± 2
35-40	124 ± 1	122 ± 3
45-50	124 ± 3	123 ± 3
60-65	124 ± 3	123 ± 3
75-80	123 ± 7	124 ± 2
90-95	125 ± 7	121 ± 3
105-110	125 ± 7	123 ± 5

Table 25: SBP (mmHg) mean values (± SEM) indicate no significant differences (*p>0.05) over the time course from mean baseline values with both cold and isothermic saline solution saline ingestion.

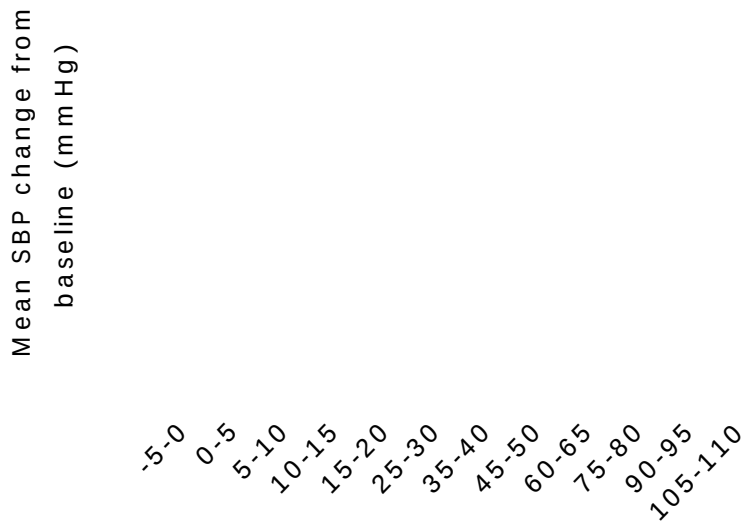

Figure 28: Mean change in SBP (mmHg) between saline at either body temperature or cold visits (± SEM) over the time course. There are no significant differences ($p>0.05$) over the time course from respective mean baseline values with both cold and body temperature saline solution. There is a 15 minute trend towards reduced mean SBP compared with mean baseline value with cold saline solution.

The assessment of the two branches of cardiac ANS show that the LF power and LF/HF ratio do not change significantly in response to isothermic saline ingestion (mean 3.41 ± 2.78 nu increase, $p>0.05$ and mean 0.13 ± 0.06 nu increase, $p>0.05$) respectively (figures 28 and 29). Nevertheless, LF power and LF/HF ratio responses to cold saline ingestion indicate significant increase in both LF power (mean 9.93 ± 3.70 nu increase, $p<0.05$) and LF/HF ratio (mean 0.27 ± 0.07 nu increase, $p<0.05$) between 5 and 30 minutes from mean baseline values.

Time (min)	LF (nu) with saline at body temperature	LF (nu) with saline at cold temperature
-5-0	14.24 ± 3.09	11.96 ± 2.88
0-5	13.93 ± 2.87	17.09 ± 3.94
5-10	17.55 ± 2.71	21.04 ± 3.74*
10-15	19.38 ± 3.27	23.91± 4.49*
15-20	18.94 ± 2.88	22.11 ± 4.08*
25-30	17.42 ± 3.22	20.48 ± 2.50*
35-40	18.20 ± 1.84	20.05 ± 1.73
45-50	18.61 ± 1.70	19.43 ± 2.14
60-65	17.68 ± 3.49	19.54 ± 1.63
75-80	17.01 ± 2.87	18.65 ± 4.65
90-95	18.20 ± 2.00	17.51 ± 3.69
105-110	17.33 ± 3.65	15.51 ± 2.21

Table 26: LF (nu) mean values (± SEM) indicate significant differences (*$p<0.05$) between 5 and 30 minutes from mean baseline value with cold saline solution. No statistical changes over time from mean baseline value with isothermic saline ingestion and no significant changes at any time point cold versus body temperature saline solution.

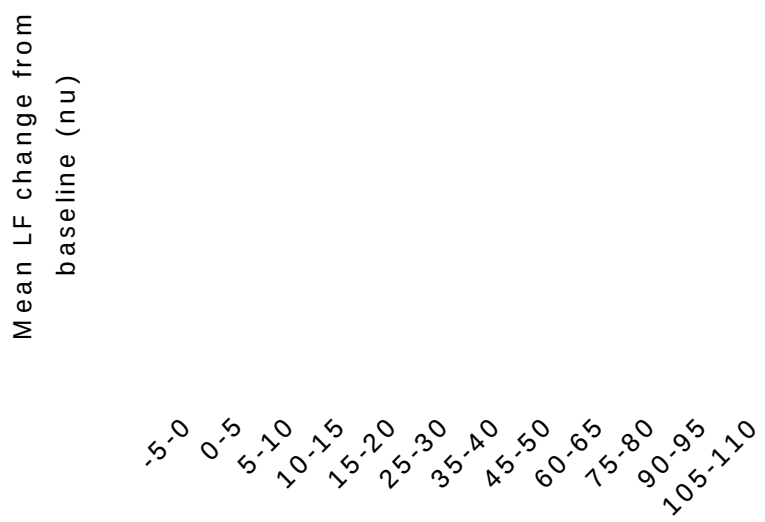

Figure 29: Mean change in cardiac interval LF power (nu) between saline at either body temperature or cold visits (± SEM) over the time course. *p< 0.05 significant differences between 5 and 30 minutes from mean baseline value with cold saline solution.

Time (min)	LF/HF ratio with saline at body temperature	LF/HF ratio with saline at cold temperature
-5-0	0.17 ± 0.04	0.15 ± 0.05
0-5	0.20 ± 0.05	0.26 ± 0.07
5-10	0.33 ± 0.12	0.36 ± 0.07*
10-15	0.33 ± 0.11	0.43 ± 0.08*
15-20	0.37 ± 0.13	0.42 ± 0.07*
25-30	0.37 ± 0.03	0.45 ± 0.06*
35-40	0.31 ± 0.02	0.32 ± 0.04
45-50	0.27 ± 0.03	0.30 ± 0.06
60-65	0.28 ± 0.09	0.28 ± 0.03
75-80	0.28 ± 0.05	0.27 ± 0.06
90-95	0.28± 0.04	0.28 ± 0.06
105-110	0.27 ± 0.04	0.28 ± 0.06

Table 27: LF/HF (ratio) mean values (± SEM) indicate significant differences (*$p<0.05$) between 5 and 30 minutes from mean baseline value with cold saline solution. No statistical differences over the time course from mean baseline value with isothermic saline ingestion and no significant changes at any time point cold versus body temperature saline solution.

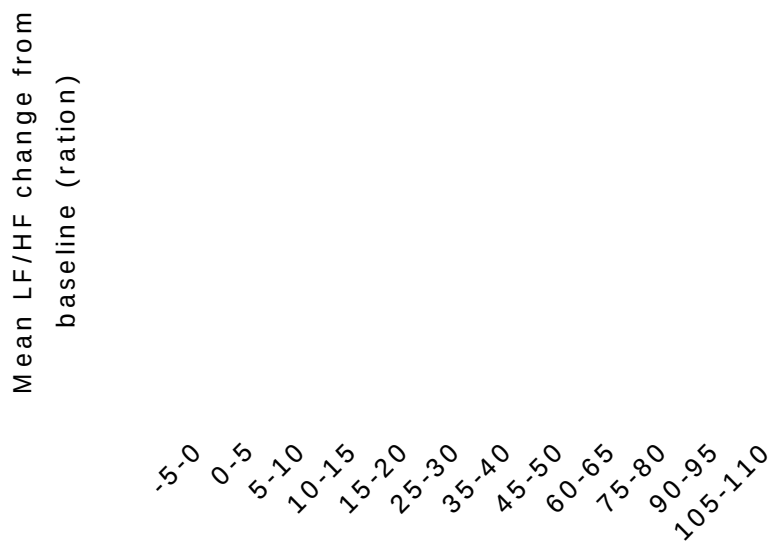

Figure 30: Mean change in cardiac LF/HF ratio between saline at either body temperature or cold visits (± SEM) over the time course. *p< 0.05 significant differences between 5 and 30 minutes from mean baseline value with cold saline solution

6. 5. Discussion

The main finding of this study was that the ingestion of 300 mL of cold isotonic saline solution invoked various cardiovascular changes, including increased cardiac vagal tone and reduced HR, contrary to the same volume of saline solution ingestion at body temperature which did not elicit any cardiovascular variations. No changes in any analysis parameter of cardiac ANS activity were shown, an observation supported by a stable HR, indicating unchanged sympathetic and parasympathetic ANS activity following saline ingestion at body temperature. The trend towards increased HR during the first 5 minutes after isothermic saline may be due to oro-pharyngeal sympathetic stimulation known to occur during the swallowing period (Endo *et al.*, 2002). Saline solution at

body temperature failed to elicit an increase in sympathetic tone observed with gastric stretch (chapter 4), probably due to the free distribution of liquid throughout the extra-and intracellular space and a small quantity of liquid which failed to induce an elongation of gastric wall and to elicit an activation of gastric stretch receptors known to activate the sympathetic nervous system (Van Orshoven *et al.*, 2004). An increase in both muscle sympathetic nerve activity and BP after gastric distension (Cottrell, 1984; Vacca *et al.*, 1996) has been shown to be directly proportional to the volume of stretch (Seth *et al.*, 2008). The unchanged cardiac autonomic activity after isothermic saline solution is in agreement with the finding from Brown *et al.*, (2005) where 7.5 ml/kg body wt (mean volume 517 ± 15 ml) of physiological (0.9%) saline ingestion did not induce changes in both vagal and sympathetic tone, and did not show any volume loading effects in young healthy subjects. These results show how the NaCL solute content in water did not have any effect on the cardiovascular activity.

As well as confirming these observations, the current study also showed an increased RMSSD between 5 and 30 minutes with cold saline drinking, providing a mechanism to explain a corresponding vagally mediated decrease in HR as it indicates an activation of cardiac parasympathetic tone known to reduce the HR (Green, 2005). The cold mediated reduced HR appeared within 5 minutes probably because during the first 5 minutes, the sympathetic activation during the swallowing period may have counteracted the vagally mediated reduced HR. This cold mediated decreased HR lasted only for 30 minutes maybe due to the intra-abdominal temperature warming up cold saline solution to body

temperature levels within a short period, consistent with another observation where ingestion of a 400 ml drink of orange juice at 4°C returned to the normal body temperature within 30 minutes (Sun *et al.*, 1988). The underlying transduction mechanisms of cold sensitivity being reported in vagal afferent nerve fibres in cultured vagal sensory neurons in animal nodose ganglion (Fajardo *et al.*, 2008), and in mammalian cell lines (Story *et al.*, 2003), it is possible that changes related to an activation of thermosensitive afferent vagal nerve fibres which were found in the stomach (El Ouazzani and Mei, 1982) may be triggered by the stimulation of gastric TRPM8 thermoreceptors (Girona *et al.*, 2014). These receptors known to be activated by temperatures below 28°C (Fajardo *et al.*, 2008), convey to the brainstem temperature-related information via vagal afferent nerve fibres (Girona *et al.*, 2014), which results in a decline in HR (Paton *et al.*, 2005; Bailey *et al.*, 2006). The current study also showed an earlier cold mediated sympathetic inhibition indicated by QTc interval shortening, followed by its reintroduction probably corresponding to the intra-abdominal temperature warming up cold saline to the body temperature levels (Girona *et al.*, 2014). A short duration in cardiac sympathetic stimulation (1-3 sec), indicating lower noradrenaline concentration and a prolongation of cardiac action potential generates a sympathetically mediated lengthening in QT interval (Arrowood *et al.*, 1993). Therefore, its inhibition induces a QT shortening. The trend towards decreased SBP due to the cold mediated sympathetic inhibition combined with a fall in HR may indicate a substantial decrease in the workload to the heart, associated with a decreased cardiac output (Scott *et al.*, 2001) after cold saline drinking. Additionally, the LF power

and the LF/HF ratio shifted toward vagal predominance, evoking stronger parasympathetic activity suppressing sympathetic tone during the first 30 minutes after cold saline ingestion. The sympathetic inhibition may occur in the nucleus of the NTS where the two afferent limbs (vagal-and sympathetic fibres) firstly meet during their central pathway (Thayer and Lane, 2009). A study performed by another group (Brown *et al.*, 2005) has indicated that 0.9% of saline ingestion at 21°C had no effect on cardiovascular autonomic regulation, contrary to the ingestion of saline solution at 6 °C observed in our experiment. The difference may be related to the difference in temperature at which saline solution was ingested. In their experiment, saline solution was served at 21°C, whereas in this experiment the solution was ingested at 6°C. It is also known that TRPM8 receptors are activated only by at temperature below 28°C (Fajardo *et al.*, 2008). Therefore, saline solution served at 21°C might have been warmed up in intra-abdominal body temperature, reaching a level at which TRPM8 receptors could not be activated. Contrarily cold saline solution served at 6°C in our experiment, may have reached the stomach at the temperature below 28°C which is known to activate TRPM8 receptors which send a vagally mediated temperature-related information to the NTS of the midbrain (Fajardo *et al.*, 2008, Girona *et al.*, 2014), which in return induced an increased cardiac vagal tone with a corresponding decreased HR.

Overall, new discoveries from this study revealed that the decreased HR after cold saline ingestion lasted only for 30 minutes, probably due to the intra-abdominal temperature warming up cold saline solution to body temperature levels within a short period. The earlier fall in HR and BP reflex response during

the increase vagal tone and sympathetic withdrawal, probably implicating the RVLM, the CVLM, and the NA with cold saline, is probably mediated by the activation of gastric TRPM8 receptors. The stimulation of these TRPM8 receptors sends through sensorial vagal neurons temperature-related information to the brainstem (Farardo *et al.*, 2008). This decrease in HR, the inhibition of sympathetic tone, and the substantial decrease in cardiac workload after cold saline drinking may also be a relevant observation in clinical use with patients with cardiovascular pathologies, although further studies are needed to address the role of stomach cooling with isotonic saline solution in human pathologies. These observations are not seen with isotonic saline at body temperature where the drinking the same amount of isothermic saline did not have any effects on the cardiovascular system, including no volume loading effects.

CHAPTER 7: EFFECTS OF WATER DRINKING AND TEMPERATURE ON CARDIAC AUTONOMIC EFFERENT ACTIVITY IN HEALTHY HUMAN VOLUNTEERS

7. 1. Introduction

The effects of water ingestion on human cardiovascular autonomic regulation may be induced by gastric distension (Routledge *et al*., 2002), volume loading effects (Andrews *et al*., 1980; Young and Mathias, 2004), or osmolality effects (Brown *et al*., 2005). Contrary to sympathetically mediated increase in both BP, and HR with gastric distension observed in the current study (chapter 4) and in the work of others (Cottrell, 1984; Van Orshoven *et al*., 2004), water ingestion is reported to elicit an increase in both sympathetic and cardio-vagal activities (Brown *et al*., 2005) with little or no change in BP (Scott *et al*., 2001; Brown *et al*., 2005), suggesting that the mechanisms underlying the cardiovascular responses to water ingestion may not be identical to those related to gastric stretch. In addition, if the pressor response after water drinking was associated with gastric distension, this pressor response would be maximal at the end of the ingestion, thereafter diminishing as water enters the duodenum (McHugh *et al*., 2010) or as it is absorbed from the stomach. However, the maximal pressor response after water infusion into the stomach of mice was not observed at the end of water infusion, a slow onset gradually built up to a maximal response at around 15-25 minutes following water infusion (McHugh *et al*., 2010), indicating that the pressor response is not associated with gastric distension. The current study has also indicated that ingestion of isotonic saline solution does not induce a pressor effect. This observation is in line with an report another study

where the intraduodenal infusion of the same volume of isotonic saline solution and water showed that water only (not saline) elicited an increase in BP in mice (McHugh *et al.*, 2010), suggesting that water hypo-osmolality (not volume loading effects) appears to be the stimulus inducing the cardiovascular responses (Brown *et al.*, 2005; McHugh *et al.*, 2010). Therefore, cardiovascular responses to water ingestion are not a result of stomach distension (Young and Mathias, 2004), nor a consequence of parasympathetic baroreceptor-mediated response to the pressor effect of cardiovascular volume-loading (Routledge *et al.*, 2002), but are associated with hypo-osmolality of water (May and Jordan, 2011). In healthy subjects, the expected hypo-osmolality mediated increase in HR due to the activation of sympathetic nervous system after water ingestion is counteracted by hypo-osmolality mediated vagal co-activation which leads to decline in HR (Routledge *et al.* 2002). The increase in peripheral vascular resistance without an increase in BP is due a compensatory reduction in cardiac output after water drinking in young healthy subjects (Scott *et al.*, 2001; Lu *et al.*, 2003). Conversely, in patients suffering from severe autonomic failure, and in elderly people (with impairment in baroreflex regulation), water drinking generates a greater pressor effect expressed by increasing BP due to a lack of a buffering mechanism (Cariga *et al.*, 2001; 2001; May and Jordan, 2011). Patients with autonomic failure show a severe orthostatic hypotension due to an interruption of the efferent arc of the baroreflex (parasympathetic and sympathetic nerves), situation similar to the administration of ganglionic blockers in healthy subjects (Shanon *et al.*, 2002). 500 mL of water drinking in patients with autonomic failure caused a substantial increase in blood pressure

by more than 30 mmHg for almost an hour (Cariga and Mathias, 2001), a mechanism used to improve orthostatic tolerance (Lu *et al*., 2003). The autonomic reflex tends to mask potential changes in BP after water drinking in healthy people (May and Jordan, 2011). The mechanism underlying the effects of water drinking on the cardiovascular system can be elucidated only by considering autonomic failure where the phenomenon is unmasked in humans or animals (Brown *et al*., 2005; McHugh *et al*., 2010).

7. 1. 1. Nature of Afferent and Efferent Limbs

Vagal and splanchnic sensory neurons are the two major pathways which transmit information from the GI tract to the CNS. However, surgically baroreflex-impaired mice that underwent bilateral sub- diaphragmatic vagotomy before water infusion, showed a similar increase in BP as intact animals indicating that vagal afferent neurons are not essential for the pressor effect of water (McHugh *et al*., 2010)**.** Increased sympathetic activity, accompanied with increased plasma noradrenaline but not with renin or vasopressin, suggests that the sympathetic nervous mechanism may be responsible for the pressor response to water drinking (Jordan *et al*., 2000). Subjects with a complete loss of sympathetic efferent function showed an absence of pressor effects after water ingestion (May and Jordan, 2011) and the pre-treatment with α_{1}-adrenoreceptor antagonist Prazosin resulted in the loss of pressor response to water ingestion in autonomic failure mice (McHugh *et al*., 2010), indicating the sympathetic efferent involvement in cardiovascular changes (May and Jordan 2011). In addition, the genetic deletion of dopamine beta- hydroxylase (the enzyme required for converting dopamine into noradrenaline), resulted in the

abolishment on the pressor response after water drinking (McHugh *et al.*, 2010), an almost completely abolished pressor response to water ingestion after interruption of ganglionic transmission by the administration of the nicotinic receptor antagonist Trimethaphan (Jordan *et al.*, 2000), and improved pressor response to water ingestion in people with vagally denervated heart in cardiac transplant (Routledge *et al.*, 2002) provide more evidence of a sympathetically efferent mediated pressor effect associated with water ingestion (McHugh *et al.*, 2010). The loss of sympathetic tone and parasympathetic function in autonomic impairment is less complete than that observed in complete ganglionic blockade, explaining the increased BP with water drinking in those subjects (May and Jordan, 2011). Therefore, sympathetic neurotransmission is considered to be the efferent limb mediating the pressor effect associated with water ingestion (McHugh *et al.*, 2010; May and Jordan, 2011). However, it has been suggested that the relative hypo-osmolality of water acts as an afferent signal via osmoreceptive nerve fibres in the gut to elicit osmopressor response (Bourque *et al.*, 2007), via both vagal and ascending spinal cord afferent nerves serving the gut, or could be limited to spinal sensory afferent nerve fibres capable of influencing directly the sympathetic output at spinal cord level (Grundy, 2002).

7. 1. 2. Pathways Mediating Sympathetic Activation with Water Ingestion

The neural pathways of pressor responses to water ingestion may include brainstem centres, including the NTS which receive splanchnic nerve fibres, or may be limited to the spinal pathway (McHugh *et al.*, 2010). The increase in BP

after water ingestion in multiple system atrophy subjects where the lesion to the efferent part is situated in the brain stem with some distal efferent sympathetic neurons being in part intact could drive sympathetic activity (Benarroch *et al.*, 2008), suggests that postganglionic sympathetic neurons can be activated by spinal reflexes (May and Jordan, 2011). Additionally, water drinking in cervical spinal cord injury where sympathetic neurons are disconnected from the brain stem though intact (May and Jordan, 2011), postganglionic sympathetic nerves fibres could still be activated by spinal reflexes instead of using the reflexes mediated via the brain stem (Tank *et al.*, 2003). These observations indicate that postganglionic sympathetic neurons can be activated by spinal reflexes but not by reflexes travelling through the brain stem, including the baroreflex (May and Jordan, 2011). Patients with cervical spinal cord transection had an intact pressor response to water drinking, contrary to sub-diaphragmatically vagotomised animals that showed a shorter pressor response than intact animals suggesting that the vagal nerve is not essential for the pressor effect to be expressed (Tank *et al.*, 2003). These observations suggest that activation of sympathetic efferent neurons by water ingestion may not involve a brainstem mechanism; instead the sympathetic efferent nerve fibres may be activated through a spinal mechanism (Jordan, 2005). However, although activation of sympathetic efferent tone is generally known to induce an increase in renin production (Robyn *et al.*, 2014), subjects who have received Captopril, the inhibitor of Angiotensin Converting Enzyme (ACE), prior to water ingestion did not show an alteration of the pressor

response (McHugh *et al.*, 2010), suggesting non-implication of RAA axis, perhaps as hormonal mechanism would be too slow. The dopamine ß-hydroxylase knockout mice with no detectable noradrenaline in blood and urine or tissue, did not produce an increase in BP in response to water ingestion (McHugh *et al.*, 2010), indicating a sympathetic nervous system mechanism being responsible for the pressor effect instead of a renin-angiotensin-aldosterone axis mechanism (Raj *et al.*, 2006).

7. 1. 3. Water Drinking and Hypo-osmolality

The decrease in portal osmolality relative to systemic osmolality after water infusion into the portal vein (May and Jordan, 2011) or following water drinking (McHugh *et al.*, 2010) and the subsequent production of a pressor effect, indicate that water hypo-osmolality may be acting via osmosensors in the portal/hepatic circulation which cause a reflex pressor response (McHugh *et al.*, 2010). This water induced extracellular hypo-osmolality is reported to be detected by TRP osmoreceptors (May and Jordan, 2011) located either in the peripheral regions, including afferent neurons, GI tract, and portal system (Brierley *et al.*, 2008), or centrally in the organum vasculosum of the lamina terminalis (OVLT) and subfornical organ (SFO) of the circumventricular organs (McKinley *et al.*, 1992) and NTS (Daniels and Fluharty, 2004). Osmotic signals can also be detected by primary chemosensory afferents (Gallego *et al.*, 1979) and by afferent fibres in the hepatic branch of the vagus nerve (Adachi *et al.*, 1976). Osmosensory input collected from the splanchnic mesentery is also relayed to central areas via ascending projections carried in part via spinal pathways (Vallet and Baertschi, 1982). The VP and OT synthesised in the

supraoptic nucleus and the paraventricular nucleus of the hypothalamus are also intrinsically osmosensitive since they activate water reabsorption by the kidney (Mason, 1980; Oliet and Bourque, 1993). The brain nuclei osmoreceptors have no blood/brain barrier and are associated with the regulation of fluid and electrolytes balance and can control thirst, sodium secretion, blood volume regulation and vasopressin secretion (McHugh *et al.*, 2010). The effect of water drinking could be related to the changes in plasma osmolality (Stricker *et al.*, 2002).

7. 1. 4. Molecular Mediators

TRPV1 and TRPV4 osmoreceptors are the potential mediators of the osmoreceptor responses associated with water ingestion (Nilius and Owsianik, 2011). TRPV1 osmoreceptors are implicated in CNS vasopressin control and have a potential role in the osmolality response to water and TRPV4 osmoreceptors have the ability to respond to a wide range of stimuli, including the water osmolality (McHugh *et al.*, 2010). However, genetic knockout TRPV1 ($TRPV1^{-/-}$) mice produced a similar response as observed in wild-types after duodenal infusion of water (Sharif *et al.*, 2006; Ciura and Bourque, 2006), indicating that TRPV1 receptors are not mediators of the pressor response to water. Contrarily, genetic knockout TRPV4 ($TRPV4^{-/-}$) mice lost the pressor response to duodenal infusion of water (Alessandri-Haber *et al.*, 2005), suggesting TRPV4 as the only candidate accepted as a mediator of osmotic response to water infusion (McHugh *et al.*, 2010; May and Jordan, 2011). TRPV4 receptors are present in different locations of the body, including the GI tract, mesenteric vessels, liver, cholangiocytes,

and dorsal root ganglia (McHugh *et al.*, 2010). Stimulation of TRPV4 receptors results in a release of various neurotransmitters such as NO, CGRP and substance P, capable of inducing different functions, including regulation of sympathetic reflexes and BP (Grant *et al.*, 2007). Nevertheless, infusion of water directly into the portal vein in TRPV4 $^{-/-}$ mice induced an immediate pressor effect that tended to be slightly higher in magnitude than duodenal infusion (20.0 ± 11.9 mmHg vs 14.9 ± 7.4 mmHg, $p > 0.05$) (McHugh *et al.*, 2010), suggesting a presence of some osmoreceptors independent to TRPV4 receptors in the portal system (May and Jordan, 2011). Therefore, there may be both TRPV4-dependent sensitive effects to physiological hypo-osmolality and TRPV4- independent mechanisms responding to more dramatic hypo-osmolality in portal system (McHugh *et al.*, 2010). TRV4-dependent osmoreceptors may be sensing the hypo-osmolality changes in mesenteric venules, but not in the portal vein and liver, whereas TRV4-independent osmoreceptors may be sensing changes in the hepato-portal system (Jensen *et al.*, 2013).

7. 2. Aim of the Experiment

Although there has been much research and progress in the study of the cardiovascular responses to water ingestion (Brown *et al.*, 2005; McHugh *et al.*, 2010; May and Jordan, 2011), there is still a lack of clarity regarding the effects of water temperature on the cardiovascular changes, even though humans tend to drink water at a temperature below room temperatures (21-22°C) (Burdon *et al.*, 2012). Effects of water ingestion have also been observed in healthy young subjects drinking about 500 mL (Brown *et al.*, 2005; Jordan *et al.*, 2000; Schroeder *et al.*, 2002; Shannon *et al.*, 2002; Scott *et al.*, 2001; Cariga *et al.*,

2001; Tank *et al*., 2003), but little is known about the cardiovascular effects of drinking a small quantity of water such as 300 mL. The aim of this study was to compare the cardiovascular responses of cold water ingestion with the same volume of water ingested at body temperature, and to evaluate whether these effects are the same in response to a small quantity of water ingestion such as 300 mL.

7. 3. Methods and Materials

Nine healthy volunteer young individuals (mean age: 23.30 ± 3.13 years) gave informed consent to take part in a protocol consisting of drinking 300 mL of still water at either 37°C or 6°C to elicit either hypo-osmolality effect or cold combined to hypo-osmolality effects respectively. One of the subjects was rejected because he failed to synchronise his breathing the timer operating at 0.2 Hz according to the experimental protocol (chap 2). This volume (300 mL) was similar to another experiment where elderly subjects (mean age 73 ± 2.0 SEM) ingested 300 mL of water with non-significant decrease in HR (Tai *et al*., 2011). Subjects were fitted with equipment for cardiac assessments (ECG), ventilation monitoring (respiratory belt transducer), and continuous BP measurement (finger plethysmograph). After a 5 minute recording session prior to water ingestion, different recordings were performed according to diverse intervals as described in chapter 2. All the requirements as outlined in chapter 2 were observed. Offline analysis was carried out from the results of the two visits to explain the effects of cardiovascular changes after water ingestion at these two different temperatures. In accordance with the general methods and materials, values of HR, cardiac interval RMSSD, QTc interval, LF power, LF/HF

ratio, and SBP are reported as changes from the average of respective baseline values. Statistical analyses were carried out using one-way ANOVA with Bonferroni post hoc testing to compare each time point over the post-drink period with the respective baseline value and paired-test to compare equal time points of the two visits.

7. 4. Results

All subjects ingested water at the two different temperatures without problems, and no one reported any sensation of discomfort, bloating, nausea or pain. No urge to void the bladder was expressed by any of the subjects during the recording period. The resting baseline of HR (bpm), RMSSD (msec), QTc interval (msec), LF power (nu), LF/HF ratio, and SBP (mmHg) did not differ significantly between cold and control sessions (tables 25-30).

Subjects responded to cold water drinking with a significant decrease in mean HR (mean 7.05 ± 2.16 beats/min decrease, $p<0.05$) between 5 and 40 minutes from mean baseline, whereas isothermic water drinking shows a no significant change in mean HR (mean 1.30 ± 2.49 beats/min reduction, $p>0.05$) over the time course from baseline values (figure 30).

Time (min)	HR (bpm) with water at body temperature	HR (bpm) with water at cold temperature
-5-0	64.62 ± 2.58	67.71 ± 2.53
0-5	65.63 ± 2.65	64.47 ± 2.49
5-10	64.62 ± 2.72	60.68 ± 1.97*
10-15	64.27 ± 2.56	60.46 ± 2.05*
15-20	64.40 ± 2.79	61.34 ± 2.37*
25-30	62.24 ± 2.00	60.08 ± 2.26**
35-40	62.89 ± 1.79	60.75 ± 2.17*
45-50	61.13 ± 1.79	61.51 ± 2.64
60-65	63.20 ± 2.79	63.79 ± 2.90
75-80	62.46 ± 2.16	62.53 ± 2.86
90-95	63.22 ± 2.20	63.10 ± 2.87
105-110	63.59 ± 3.10	63.56 ± 3.12

Table 28: HR (bpm) mean values (± SEM) indicate significant differences (*$p<0.05$, **$p<0.01$) with water ingestion at 6°C between 5 and 40 minutes from mean baseline value. No significant changes over time from mean baseline value with water ingestion at 37°C.

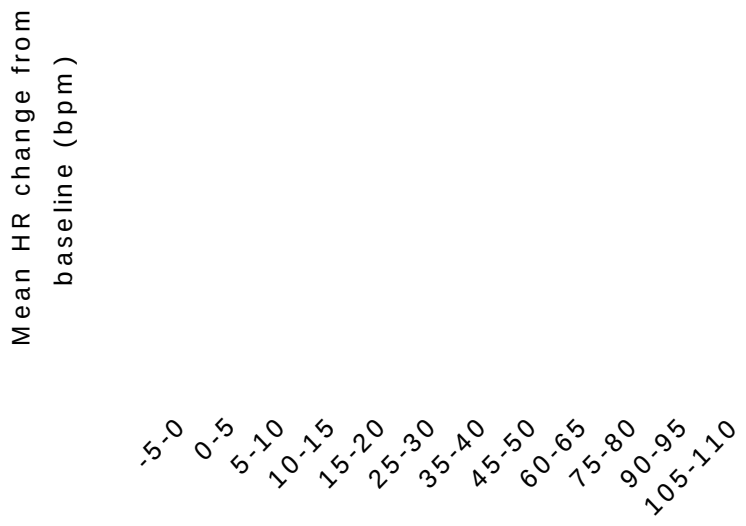

Figure 31: Mean change in HR (bpm) between water ingestion at body temperature and cold visits (± SEM) over the time cours. *p <0.05, **p<0.01, significant differences over time from mean baseline value with cold water. Significant changes (p<0.05) at each time point cold versus isothermic water ingestion between 5 and 40 minutes.

The assessment of cardiac interval vagal activity indicates that ingestion of cold water induced an immediate significant increase in mean cardiac interval RMSSD (mean 34.50 ± 20.93 msec increase, p< 0.05) which was sustained for 40 min (figure 31). There is also a significant increase in mean RMSSD (mean 23.95 ± 20.50 msec increase, p<0.05) between 5 and 40 minutes from mean baseline value with water ingestion at body temperature.

Time (min)	RMSSD (msec) with water at body temperature	RMSSD (msec) with water at cold temperature
-5-0	94.67 ± 17.94	87.72 ± 16.07
0-5	98.36 ± 18.28	110.42 ± 18.72*
5-10	114.15 ± 18.47*	119.52 ± 19.20*
10-15	115.79 ± 19.22*	122.28 ± 21.85*
15-20	118.87 ± 19.79*	126.69 ± 21.98*
25-30	122.50 ± 20.12*	126.26 ± 21.86**
35-40	122.82 ± 20.54*	125.14 ± 21.95*
45-50	117.99 ± 18.44	113.40 ± 18.71
60-65	107.85 ± 19.99	104.95 ± 19.65
75-80	112.17 ± 19.07	103.19 ± 21.40
90-95	109.30 ± 19.10	95.95 ± 21.71
105-110	108.21 ± 20.69	93.26 ± 24.45

Table 29: RMSSD (msec) mean values (± SEM) show significant differences (*p<0.05, *p<0.01) during the first 40 minutes from mean baseline values with water ingestion at either 6˚C or 37˚C. No significant changes at any time point cold versus isothermic water drinking.

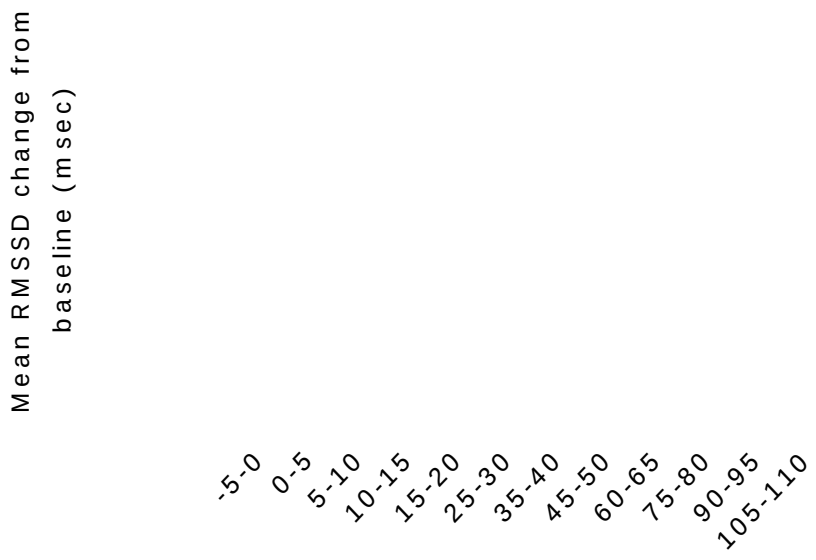

Figure 32: Mean change in cardiac interval RMSSD (msec) between water drinking at body temperature and cold visits (± SEM) over the time course. *p< 0.05, **p<0.01 significant differences over time from mean baseline values with water at either body temperature or cold. No significant changes at any time point cold versus isothermic water drinking.

However, the evaluation of sympathetic activity after water ingestion at body temperature shows a significant increased QTc interval (mean 9.86 ± 8.59 msec increase, p< 0.05), lasting for 40 minutes. However, cold water ingestion shows a short-lived significant decrease in mean QTc from mean baseline (mean 7.13 ± 10.38 msec decrease, p<0.05) during the first 5 minute, followed by its gradual increase towards the baseline level (figure 32).

Time (min)	QTc interval (msec) with water at body temperature	QTc interval with water at cold temperature
-5-0	389.94 ± 8.05	385.98 ± 9.71
0-5	395.99 ± 7.78	377.85 ± 9.93*
5-10	400.59 ± 7.89*	379.76 ± 9.51
10-15	398.48 ± 8.64*	380.26 ± 8.68
15-20	399.35 ± 9.05*	379.76 ± 8.59
25-30	400.41 ± 8.64*	384.25 ± 9.81
35-40	399.48 ± 9.33*	384.00 ± 9.71
45-50	395.63 ± 8.79	385.68 ± 11.25
60-65	395.60 ± 8.99	384.79 ± 10.74
75-80	394.16 ± 8.21	385.57 ± 10.67
90-95	395.14 ± 8.93	386.80 ± 10.81
105-110	394.47 ± 9.77	387.90 ± 10.96

Table 30: QTc interval (msec) mean values (± SEM) indicate significant differences (*$p<0.05$) with water ingestion at ingestion at either 6 °C or 37°C over time from mean baseline value.

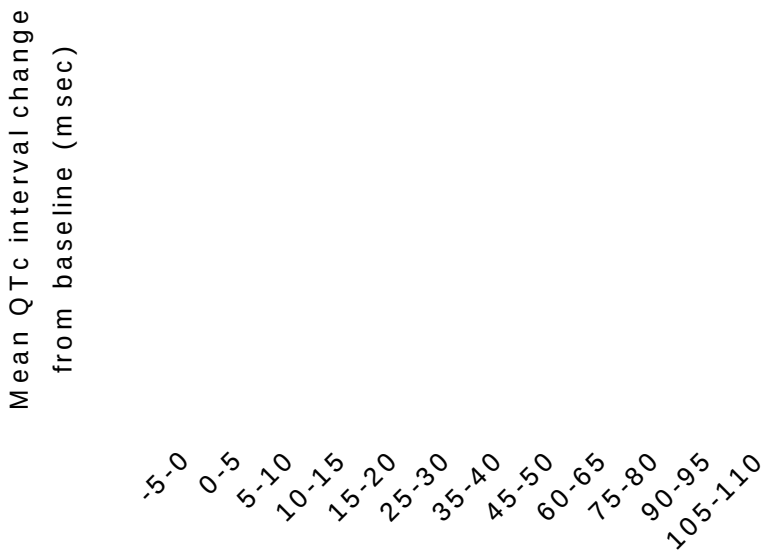

Figure 33: Mean change in QTc interval (msec) between water drinking at body temperature and cold visits (± SEM) over the time course. *p< 0.05 significant differences over time from mean baseline values with isothermic water and cold ingestion.

Furthermore, although water drinking at body temperature induced a trend towards increased SBP (mean 2.80 ± 5.35 mmHg increase, p>0.05) over the time course, there is a 15 minute cold mediated trend towards decreased SBP (mean 3.60 ± 5.42 mmHg decrease, p> 0.05) with cold water ingestion (figure 33).

Time (min)	SBP (mmHg) with water at body temperature	SBP (mmHg) with water at cold temperature
-5-0	118 ± 6	118 ± 6
0-5	122 ± 5	121 ± 7
5-10	120 ± 6	114 ± 6
10-15	119 ± 6	115 ± 5
15-20	120 ± 5	115 ± 5
25-30	122 ± 5	122 ± 3
35-40	123 ± 6	124 ± 1
45-50	122 ± 5	124 ± 3
60-65	123 ± 4	122 ± 3
75-80	122 ± 5	123 ± 3
90-95	122 ± 5	122 ± 4
105-110	119 ± 4	122 ± 4

Table 31: SBP (mmHg) mean values (± SEM) indicate no significant differences ($p>0.05$) with water ingestion at either 6˚C or 37˚C over the time course from mean baseline values. No significant changes at any time point cold versus isothermic water ingestion.

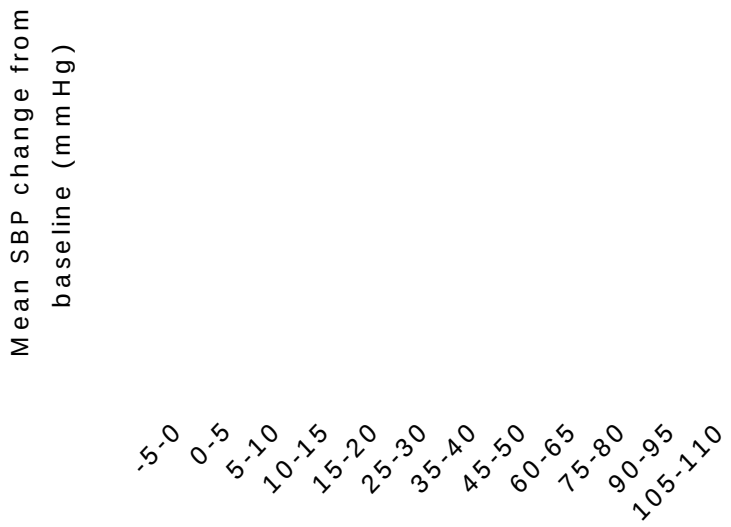

Figure 34: Mean change in SBP (mmHg) between water ingestion at body temperature and cold visits (± SEM) over the time course. No significant changes in mean SBP at both temperatures over the time course from mean baseline value. However, there is a 15 minute trend towards decreased SBP with cold water.

Finally, cardiac interval LF power shows no significant changes with water ingestion at both body temperature (mean 2.46 ± 2.85 nu increase, $p>0.05$), and cold temperature (mean 3.04 ± 2.81 nu increase, $p> 0.05$) over the time course (figure 34). Likewise, cardiac interval LF/HF ratio does not indicates any significant changes in response to either cold water (mean 0.03 ± 0.04 increase, $p> 0.05$) or water at body temperature (mean 0.19 ± 0.04 increase, $p> 0.05$) over the time course (figure 35).

Time (min)	LF (nu) with water at body temperature	LF (nu) with water at cold temperature
-5-0	13.19 ± 2.49	14.34 ± 3.44
0-5	13.52 ± 2.93	16.53 ± 2.93
5-10	15.04 ± 3.41	16.15 ± 3.34
10-15	14.77 ± 2.85	17.51 ± 4.19
15-20	16.87 ± 2.75	18.33 ± 3.38
25-30	15.25 ± 2.11	16.84 ± 2.67
35-40	16.24 ± 2.50	17.92 ± 2.58
45-50	16.28 ± 2.60	18.64 ± 2.81
60-65	17.81 ± 2.70	17.11 ± 2.86
75-80	15.78 ± 3.27	18.14 ± 2.29
90-95	14.69 ± 1.90	16.34 ± 1.97
105-110	14.92 ± 2.70	17.64 ± 1.87

Table 32: LF (nu) mean values (± SEM) indicate no significant differences ($p>0.05$) with water ingestion at either 6°C or 37°C over the time course from mean baseline values. No significant changes at any time point cold versus isothermic water ingestion.

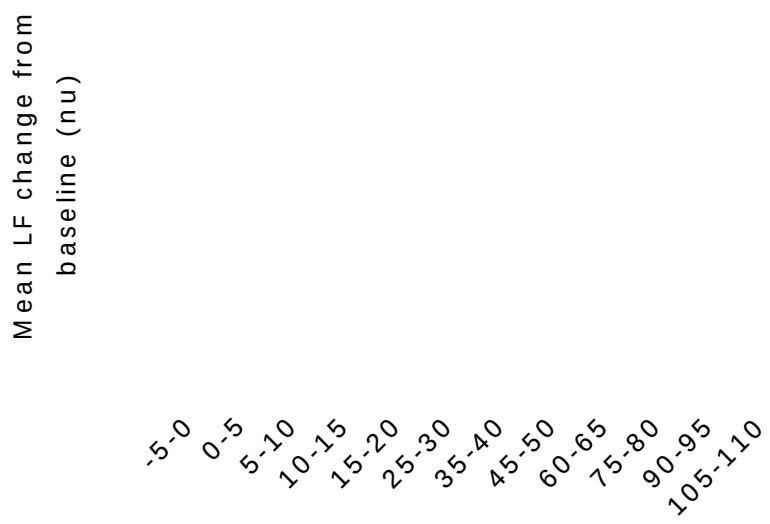

Figure 35: Mean change in cardiac interval LF power (nu) between water ingestion at body temperature and cold visits (± SEM) over the time course. No significant changes in mean LF power at both temperatures over the time course from mean baseline value. No significant changes at any time point cold versus isothermic water ingestion.

Time (min)	LF/HF ratio with water at body temperature	LF/HF ratio with water at cold temperature
-5-0	0.16 ± 0.05	0.15 ± 0.03
0-5	0.15 ± 0.05	0.19 ± 0.04
5-10	0.16 ± 0.04	0.18 ± 0.04
10-15	0.17 ± 0.05	0.16 ± 0.04
15-20	0.19 ± 0.05	0.18 ± 0.04
25-30	0.18 ± 0.03	0.19 ± 0.04
35-40	0.22 ± 0.04	0.17 ± 0.03
45-50	0.22 ± 0.03	0.20 ± 0.03
60-65	0.20 ± 0.02	0.18 ± 0.02
75-80	0.17 ± 0.03	0.18 ± 0.04
90-95	0.19 ± 0.03	0.19 ± 0.04
105-110	0.21 ± 0.05	0.18 ± 0.04

Table 33: LF/HF (ratio) mean values (± SEM) indicate no significant differences ($p>0.05$) with water ingestion at either 6˚C or 37˚C over the time course from mean baseline values. No significant changes at any time point cold versus isothermic water ingestion solution.

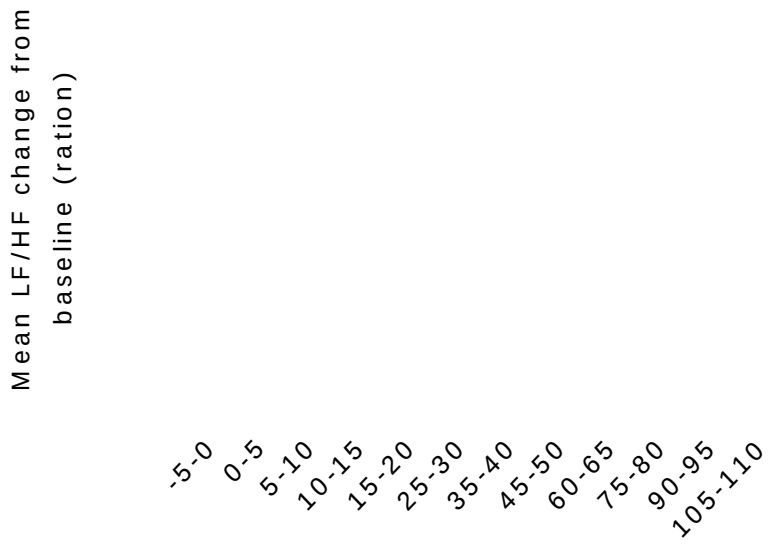

Figure 36: Mean change in cardiac interval LF/HF ratio between water ingestion at body temperature and cold visits (± SEM) over the time course. No significant changes in mean LF/HF ratio at both temperatures over the time course from mean baseline value. There are no significant differences at any time point cold versus body temperature water drinking.

7. 5. Discussion

This study indicates differential cardiovascular responses to water ingestion at different temperatures in young healthy subjects. The main finding of this study was that cold water drinking induced a decrease in HR (mean 7.05 ± 2.16 beats/min decrease, $p<0.05$) between 5 and 40 minutes, whereas water ingestion at body temperate did not evoke any significant changes over the time course (mean 1.30 ± 2.49 beats/min reduction, $p>0.05$). This suggests different mechanisms underlying the cardiovascular responses to water ingestion at the two different temperatures. There was an increase in cardiac vagal tone after water ingestion at both temperatures for about 35-40 minutes

as indicated by increase in mean cardiac interval RMSSD (with both temperatures) and a corresponding decrease in HR with cold water, but not with water at body temperature. These results are consistent with the observations from another study that 500 mL of cooled tap water (18˚C) drinking activates the cardiac vagal nerve, resulting in a declining HR from 67.6 ± 2.0 (mean ± S.E.M.) to 60.7 ± 2.4 beats/min ($P<0.01$) (Routledge *et al.*, 2002). The results were further confirmed by another finding were young healthy subjects showed a decreased HR for about 4 beats/minute after 517 ± 15 mL of water drinking at 21°C (Brown *et al.*, 2005). Besides confirming these findings, the study reported here shows an initial significant decrease in QTc interval, suggesting a sympathetic inhibition during the 5 first minutes after cold water ingestion, followed by its gradual reintroduction, probably due to intra-abdominal temperature warming of the cold water within a short period (Girona *et al.*, 2014). The combination of trend towards decreased SBP with a fall in HR may indicate a substantial decrease in the workload of the heart after cold water drinking. The underlying transduction mechanisms of cold sensitivity being reported in vagal afferent nerve fibres in mammalian cell lines (Story *et al.*, 2003), indicate that changes associated with a stimulation of thermosensitive sensory afferent vagal neurons may be triggered by the stimulation of gastric TRPM8 thermoreceptors (Girona *et al.*, 2014), known to be activated by temperature below 28˚C (Story *et al.*, 2003, Fajardo *et al.*, 2008). In addition, TRPV4 receptors are known to transmit information on osmolality (Brown *et al.*, 2005), and had also been argued to convey temperature-related information operating between 25 and 34˚C to the

brainstem (Watanabe *et al.*, 2002; Guler *et al.*, 2002). These TRPV4 may be stimulated after cold water has been warmed up within a short time to intra-abdominal environmental temperature levels (Girona *et al.*, 2014). Gastric osmoreceptor TRPV4 stimulation could potentially invoke responses by even a small quantity of water ingestion (Brown *et al.*, 2005) such as 300 mL, suggesting that local activation of TRPV4 osmoreceptors could generate cardiovascular changes even without any major changes in overall plasma osmolality. Afferent sensory neurons then convey to the NTS temperature-related information (Girona *et al.*, 2014), which results in a decline in HR. A study performed by another group (Girona *et al.*, 2014), has indicated that cold water ingestion (3°C) resulted in an immediate significant fall in HR for about 30-45 min (approximately 5 beats/min decrease), whereas this experiment showed a decrease in HR for 40 minutes after ingestion of water at 6°C. The time and amplitude differences may be related not only to the difference in temperature at which the water was ingested, but may also be associated with the position in which the experiment was performed. In their experiments, subjects were recorded while seating, whereas in the current study, subjects were assessed in semi-supine position. The upright position appears to unload arterial baroceptors and diminishes the baroreflex drive on vagal motor neurons (see 3.1 subtitle), inducing a reduction in RSA amplitude due to a shift in sympatho-vagal balance (Papegaaij *et al.*, 2014). Therefore, beside the known influence of hypo- osmolality on autonomic function (Brown *et al*, 2005; May and Jordan, 2011), the temperature of water affects cardiac vagal tone.

Contrarily, subjects responded to isothermic water ingestion with a 35-40 minute increase in both cardiac interval RMSSD and QTc interval, indicating a hypo-osmolality mediated activation of both the vagal (Routledge *et al.*, 2002) and sympathetic (Scott *et al.*, 2001) branches of cardiovascular autonomic regulation respectively. These findings are in line with previous studies that water drinking activated both the sympathetic and vagal branches of cardiovascular autonomic regulation (Brown *et al.*, 2005; McHugh *et al.*, 2010; May and Jordan, 2011). The unchanged HR observed in this experiment despite cardiac vagal activation after isothermic water ingestion may be related to hypo-osmolality mediated sympathetic activation which may have counteracted the concomitant enhanced vagal activity. These results are consistent with the report from another study where 500 mL of tap water was ingested at body temperature did not show changes in HR (Girona *et al.*, 2014). The unchanged SBP after water drinking at body temperature, a finding consistent with reports from other observations (Jordan *et al.*, 2000; Scott *et al.*, 2001; Brown *et al.*, 2005; May and Jordan, 2011), may be due to the hypo-osmolality mediated cardiac vagal activation, counteracting the effects of the hypo-osmolality mediated co-increased sympathetic tone (Schroeder *et al.*, 2002). The absence of a pressor response with the same quantity of isotonic saline ingestion at body temperature seen in our study (chapter 6), in agreement with other findings (Brown *et al.*, 2005; May and Jordan, 2011), indicates that cardiovascular responses to 300 mL of water drinking are not influenced by volume loading effects probably due to free distribution of water throughout the extra-and intracellular space (McHugh *et al.*, 2010). In addition, the autonomic

response to water drinking, consisting of simultaneous sympathetic and vagal activation would explain the lack of change in LF power and LF/HF ratio (at both temperatures), considered to reflect sympatho-parasympathetic activation (Pal *et al.*, 2014) and sympathovagal balance (Vecchia *et al.*, 2014) respectively. These results are in line with the observation from another experiment where water drinking at 21°C did not generate significant changes in both LF power and LF/HF ratio, indicating no shift in sympathovagal balance of the two branches of the ANS as water hypo-osmolality induces a simultaneous co-activation of cardiac sympathetic and parasympathetic activities (Brown *et al.*, 2005).

Therefore, beside the published effects of hypo- osmolality on cardiac autonomic functions after water drinking (Brown *et al.*, 2005, McHugh *et al.*, 2010; May and Jordan, 2011), the temperature of water influences cardiac autonomic response. In addition, the use of QTc interval as a non-invasive tool to assess the sympathetic tone (Kenigsberg *et al.*, 2007), has indicated that the sympathetic activity may be inhibited while the vagal activity is enhanced by cold water ingestion. The activation of both gastric TRPM8 and TRPV4 receptors after cold water ingestion could deliver through sensory vagal nerve fibres temperature-related information to the brainstem (Johnson *et al.*, 2009, Girona *et al.*, 2014). However, isothermic water ingestion may transmit hypo-osmotic response to the brainstem via NTS, or may use spinal reflex pathway (McHugh *et al.*, 2010) to elicit cardiovascular responses to water drinking, including the increase in both sympathetic and parasympathetic activities. The cardiovascular variations may be elicited even by a small quantity of water ingestion such as

300 mL, which may stimulate local osmoreceptors neurons located in the GI tract (Stricker *et al*., 2002).

In conclusion, this experiment shows that cold combined with hypo-osmolality effects may induce a vagally mediated increase in cardiac parasympathetic autonomic tone, with a concomitant earlier sympathetic inhibition, leading to a decrease in HR. The hypo-osmolality mediated sympathetic reintroduction may have started within 5 minutes probably due to intra-abdominal temperature warming up cold water which could allow TRPV4 receptors to be activated in order to contribute with TRPM8 receptors to the delivery through sensorial vagal nerve fibres temperature-related information to the brainstem (Fajardo *et al*, 2008, Johnson *et al*., 2009, McHugh *et al*., 2010). Around 40 minutes later, the sympathetic reactivation may have balanced the parasympathetic tone, leading the cardiovascular parameters to return near the resting level. However, hypo-osmolality effect only generated an increase in cardiovagal tone which probably contributed to buffer the hypo-osmolality mediated sympathetic co-activation, leading to no change in SBP and HR in our healthy young subjects. The cardiac sympathovagal coactivation being observed in both 300 mL (seen in this experiment) and in 500 mL of water ingestion (Brown *et al*, 2005; McHugh *et al*., 2010), this suggests that the cardiovascular to water ingestion may be induced even by a small quantity of water, which may activates the TRPV4 located in the GI tract.

CHAPTER 8: COMPARISON BETWEEN THE EFFECTS OF COLD ISOTONIC SALINE SOLUTION AND EFFECTS OF COLD WATER INGESTION ON CARDIAC AUTONOMIC EFFERENT ACTIVITY IN HEALTHY HUMAN VOLUNTEERS

8. 1. Introduction

Stimulation of TRPM8 receptor, a menthol sensitive receptor which also responds to innocuous cool temperature in the range 15-30°C (McCoy *et al.*, 2011; Raddatz *et al.*, 2014), generates various cardiovascular responses, including changes in both HR and BP (Baibars *et al.*, 2012; Meamarbashi *et al.*, 2014). The results from our experiments showed a short-lived decrease in HR with both gastric stretch combined with cold, and peppermint ingestion via the activation of TRPM8 receptors known to convey through sensorial vagal nerve, temperature-related inputs to the NTS of the brainstem (Johnson *et al.*, 2009; Girona *et al.*, 2014). Nevertheless, the comparison of their respective data could not be carried out as the two experiments used two different populations. However, the same subject population undertook both saline and water experiments, this enables us to make direct comparison to determine the time-based cardiovascular responses to cold saline and cold water ingestion.

8. 2. Data Presentation

Data presented in this chapter are extracted from our previous experiments assessing the cardiovascular responses to cold saline ingestion (cold effect only) and to cold water drinking (cold combined with hypo-osmolality effects). Subjects responded to the cold effect only with a significant decrease in mean

HR (mean 4.30 ± 1.57 beats/min decrease, $p<0.05$), between 5 and 30 minutes from the mean baseline value in response to cold saline ingestion (figure 36). Curiously, the significant decrease in HR induced by cold water lasted for a longer period between 5 and 40 minutes (mean 7.05 ± 2.16 beats/min decrease, $p<0.05$), with a greater magnitude in mean HR reduction compared with cold effect only (7.05 beats/min versus 4.30 beats/min). These effects appeared after the first 5 minutes.

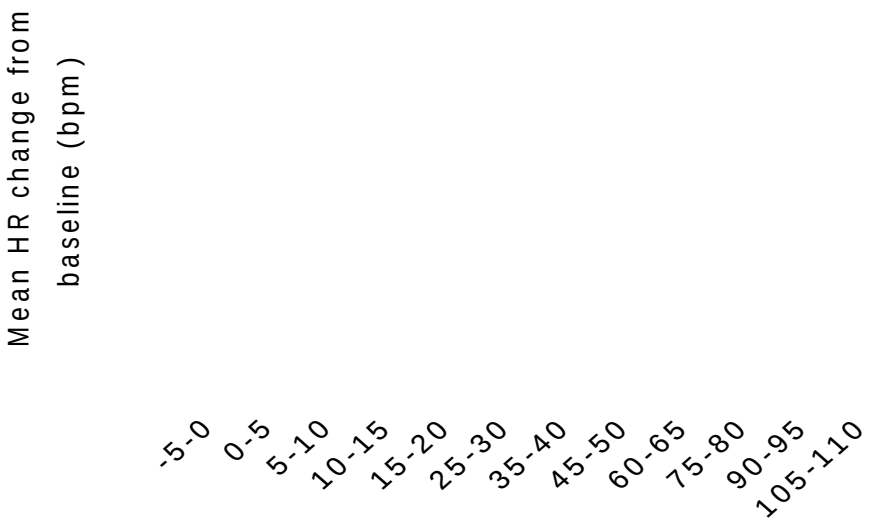

Figure 37: Mean change in HR (bpm) between cold water and cold isotonic saline solution (± SEM) over the time course. *$p < 0.05$, **$p<0.01$ significant differences over time from baseline values with cold saline and cold water. No significant changes at any time point cold saline versus cold water.

The assessment of cardiac vagal activity after cold saline ingestion indicated a significant increase in mean RMSSD (mean 29.65 ± 25.17 msec increase, $p<0.05$) from the mean baseline value between 5 and 30 minutes. Cold water ingestion generated an immediate significant increase in cardiac RMSSD (mean 34.50 ± 20.93 msec increase, $p< 0.05$), which lasted for 40 minutes Figure 37).

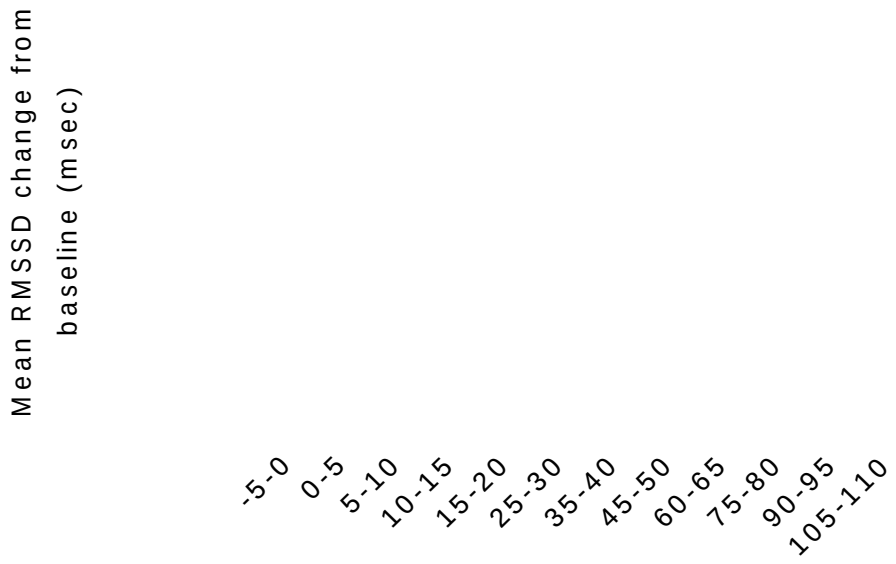

Figure 38: Mean change in cardiac interval RMSSD (msec) between cold water and cold isotonic saline solution (± SEM) over the time course. *$p<0.05$, **$p<0.01$ statistically significant differences over time from baseline values with cold saline and cold water. No significant changes at any time point cold saline versus cold water.

In addition, the investigation of cardiac sympathetic tone with cold temperature only induced a significant decrease in mean QTc interval (mean 8.11 ± 5.39 msec decrease, $p<0.05$) between 5 and 15 minutes from mean baseline value (figure 38), whereas cold combined with hypo-osmolality generated a short-lived significant decrease in mean QTc from mean baseline (mean 7.13 ± 10.38

msec decrease, p<0.05) which lasted only for 5 minutes immediately subsequent to the ingestion.

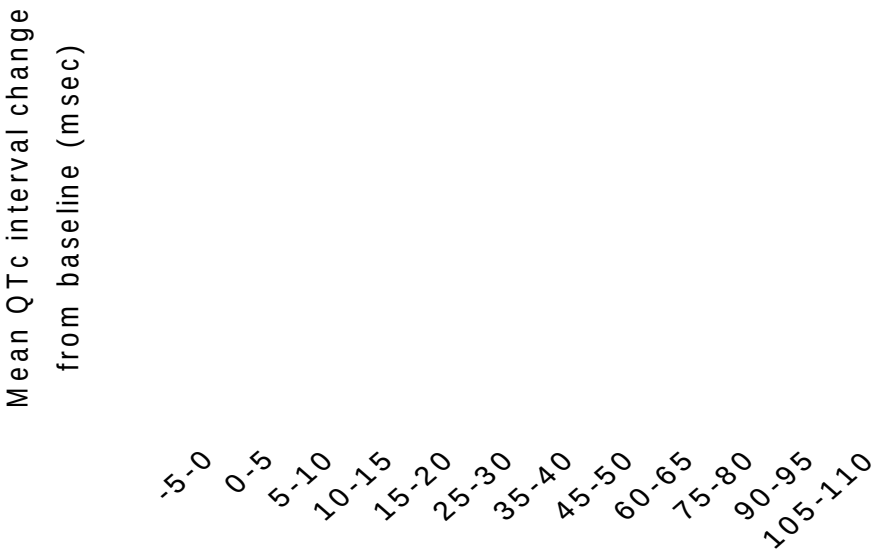

Figure 39: Mean change in QTc interval (msec) between cold water and cold isotonic saline solution (± SEM) over the time course. *p<0.05 significant differences over time from baseline values with cold saline and cold water. No significant changes at any time point cold saline versus cold water.

Furthermore, subjects showed a short-lived trend towards reduced mean SBP from mean baseline values with cold effect only (mean 3.52 ± 3.14 mmHg decrease, p>0.05) which lasted for 15 minutes. However, cold combined with hypo-osmolality effect indicated a trend towards increased SBP during the first 5 minutes (mean 2.49 ± 6.88 mmHg increase, p>0.05), followed by a slight decrease for about 15 minutes (mean 3.95 ± 5.84 mmHg decrease, p> 0.05), and then it increased slightly during the remaining period.

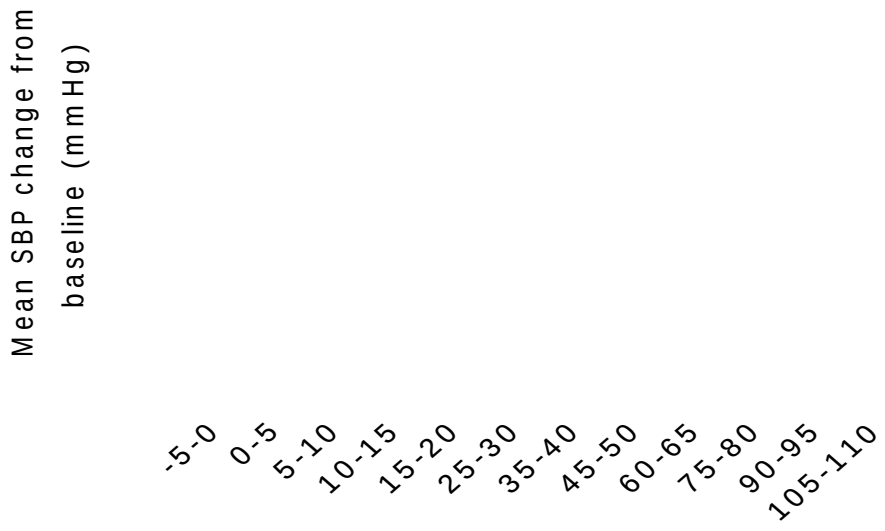

Figure 40: Mean change in SBP interval (mmHg) between cold water and cold isotonic saline solution (± SEM) over the time course. No significant differences over time from baseline values with cold saline and cold water.

The decreased HR in response to cold effect only and to cumulative cold and hypo-osmolality effects appeared after the first 5 minutes and lasted for 30 minutes with cold effect, but was sustained for 40 minutes with cumulative effects. Therefore, evaluation of cardiovascular responses with either cold effect or cold combined with hypo-osmolality effects during the first 5 minutes or between 35 and 40 minutes compared with mean of equal time points of saline at body temperature (control) shows that cold effect (figure 40) induces a slight decrease in HR either during the first 5 minutes (mean 1.55 ± 1.91 beats/ min decrease, $p>0.05$) or between 35-40 minutes (mean 3.83 ± 1.22 beats/min decrease $p>0.05$) from equal time points of respective control values. There is also a slight decrease in HR during the first 5 minutes (mean 3.24 ± 2.49 beats/minutes decrease, $p>0.05$) and between 35 and 40 minutes (mean 6.96

± 2.17 beats/minutes decrease, p>0.05) with cumulative effect, from respective equal time points of control values.

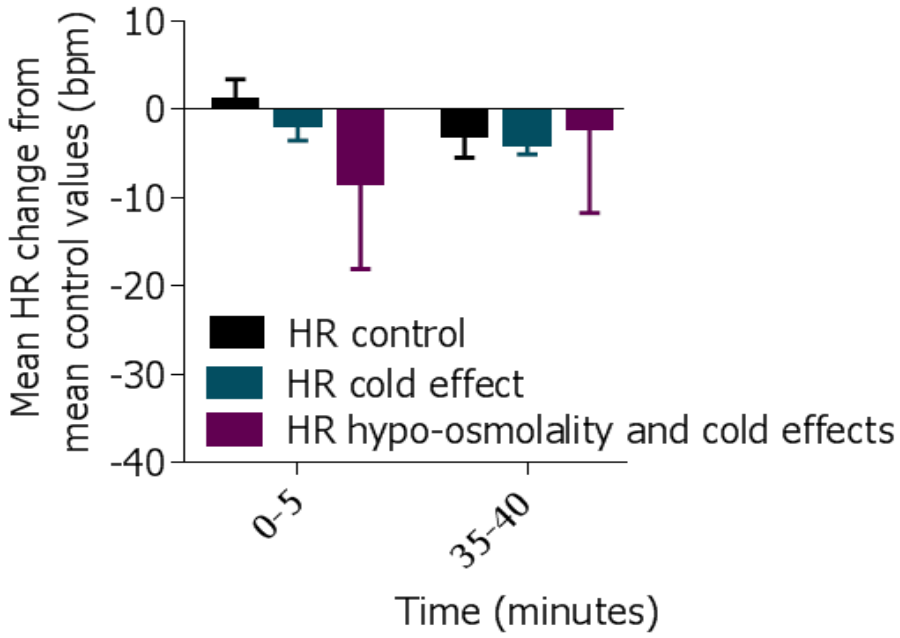

Figure 41: Variations of HR (bpm) with either cold effect or cold and hypo-osmolality effects between 0 and 5 and between 35 and 40 min. Both cold and cumulative cold and hypo-osmolality effects induce slight decrease in HR between 0 and 5 minutes and between 35 and 40 minutes from their respective equal time points of control values.

The assessment of cardiac interval RMSSD (figure 41) during the first 5 minutes and between 35 and 40 minutes indicates a trend towards increased cardiac interval RMSSD during the first 5 minutes (mean 16.25 ± 21.09 msec increase, p>0.05) and between 35 and 40 minutes (mean 25.62 ± 24.17 msec increase, p>0.05) from respective equal time points of control values with cold effect. In addition, cold and hypo-osmolality effects also regenerated a trend towards increased cardiac RMSSD during the first 5 minutes (mean 22.15 ± 18.72 msec

increase, p>0.05) and between 35 and 40 minutes (mean 37.43 ± 21.95 increase, p>0.05) from respective equal time points of control values.

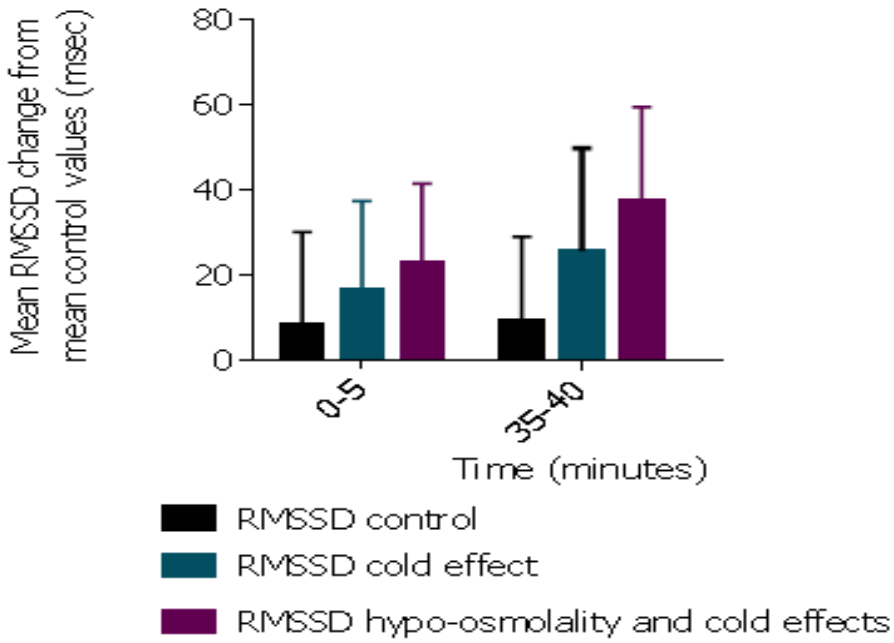

Figure 42: Variations of RMSSD (msec) with cold and combined effects between 0 and 5 and between 35 and 40 min. Both cold and cumulative cold and hypo-osmolality effects induce slight increase in HR between 0 and 5 or between 35 and 40 minutes from their respective equal time points of control values.

The evaluation of QTc interval (figure 42) indicates that cold effect induces a trend towards decreased mean QTc interval during the first 5 minutes (mean 5.13 ± 5.30 msec decrease, p>0.05) or between 35-40 minutes (mean 6.15 ± 5.19 msec decrease, p>0.05), from respective equal time points of control values. Likewise, cumulative cold and hypo-osmolality effect also showed a trend towards decreased mean QTc interval during the first 5 minutes (mean 8.24 ± 9.93 msec decrease, p>0.05) and between 35 and 40 minutes (mean 1.98 ± 9.71 msec decrease, p>0.05).

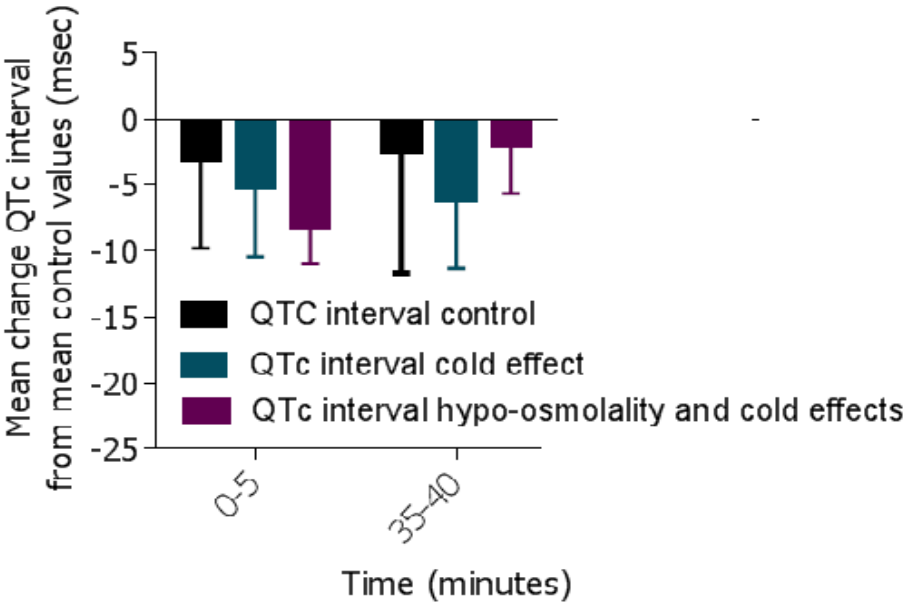

Figure 43: Variations QTc interval (msec) with either cold or combined cold and hypo-osmolality effects between 0 and 5 and between 35 and 40 min. Both cold and cumulative cold and hypo-osmolality effects induce a slight decrease in QTc interval between 0 and 5 or between 35 and 40 minutes from their respective equal time points of control values.

8. 3. Discussion

This study showed various cardiovascular responses to cold stimulus (cold saline) and combined cold and hypo-osmolality stimuli (cold water) in young healthy subjects. The main finding of this analysis was that cold effect only induced an increase in cardiac vagal tone between 5 and 30 minutes, with a corresponding decreased HR. However, combined cold and hypo-osmolality effects generated a decrease in HR between 5 and 40 minutes associated with a proportional increased cardiac vagal tone, suggesting different transduction mechanisms underlying the cardiovascular responses to the above two effects. Our findings (chapter 7) have indicated that the ingestion of water at body

temperature induced a hypo-osmolality mediated increase in both cardiac sympathetic and parasympathetic activities, which led to unchanged HR. Nevertheless, HR reduction induced by cold water ingestion may be due to a cold mediated enhancement of cardiac vagal tone, which may have inhibited the hypo-osmolality mediated increased sympathetic tone. The decreased HR with cumulative effects lasted longer (40 min) than HR reduction with cold effect only which lasted only for 30 minutes, probably due water hypo-osmolality effect known to enhance cardiac vagal tone which may have generated an additive effect to cold mediated vagal activation. Decreased HR for 30 minutes with cold effect only may correspond to the time the cold saline was warmed up to intra-abdominal temperature levels. The combined effects with cold water may explain the greater decreased in HR (mean 7.05 ± 2.16 beats/min decrease), compared with the reduction in HR with cold effect only (mean 4.30 ± 1.57 beats/min decrease). The underlying transduction mechanisms of cold effect is associated with the stimulation of gastric TRPM8 thermoreceptors (Girona *et al.*, 2014) which send temperature-related inputs to the brainstem via vagal afferent nerve fibres in order to decrease the HR (Fajardo *et al.*, 2008). In addition, TRPV4 osmoreceptors known to be activated by water hypo-osmolality (Brown *et al.*, 2005), are also reported to convey temperature related-information operating between 25-34°C to the NTS (Watanabe *et al.*, 2002), after cold water has been warmed up to intra-abdominal temperature levels (Girona *et al.*, 2014), giving a combined effect on HR reduction observed with cold water ingestion. In addition, our previous findings (chapters 6 and 7) have shown that both cold saline and cold water

ingestion did not generate the volume loading effects, due to the free distribution of the liquid (saline and water) in the extracellular and intracellular spaces, in agreement with findings from another observation (Brown *et al.*, 2005) where no volume loading effects were observed with an ingestion of almost half a litre of either water or saline solution. The cold mediated sympathetic inhibition with both cold and combined stimuli, as indicated by the QTc shortening was followed by its reintroduction probably after the intra-abdominal temperature has warmed up cold solutions within a short period (Girona *et al.*, 2014). That period was shorter with cumulative effect (5 minutes) maybe due the concomitant hypo-osmolality mediated parasympathetic stimulation which may contribute to the sympathetic inhibition, compared with cold effect only which inhibited the sympathetic activity for about 10-15 minutes. The cold mediated sympathetic inhibition with either cold effect only or cold combined with hypo-osmolality effects may be responsible for the corresponding earlier slight decreased SBP. The effects of both cold and cold combined with hypo-osmolality effect on HR appeared after the first 5 minutes, probably due to the oro-pharyngeal sympathetic activation during the swallowing period known to occur within the 5 minutes (Endo *et al.*, 2002).

The slight decrease in HR during the first 5 minutes and between 35-40 minutes with cold effect from their respective equal time point control values were probably due to a cold mediated enhancement known to reduce HR. This cold mediated vagal tone inhibited the hypo-osmolality sympathetic tone after cold water drinking and also led to a slight decrease in HR during the first 5 minutes and between 35- 40 minutes.

Overall, our data indicate that cold effect only caused a vagally mediated increase in cardiac parasympathetic autonomic tone up to 30 minutes, with a corresponding decrease HR, probably due to the intra-abdominal temperature warming up cold saline solution to body temperature levels within that period. Contrary, the period of decreased HR was lengthened up to 40 minutes with cold water ingestion, maybe due to the combined cold and hypo-osmolality effects. This contrasts other studies on autonomic failure patients (Jordan *et al.*, 2000; May and Jordan, 2011) where water temperature had no effects on cardiovascular response because of impairment of vagal afferent nerve supposed to convey temperature-related information to the brainstem.

CHAPTER 9: GENERAL DISCUSSION AND CONCLUSION

9.1. Introduction

In this thesis, time and frequency domain measures of HRV, QTc interval analysis, and blood pressure measurements were utilised to investigate the effects of gastric and homeostatic autonomic afferent reflexes on cardiac autonomic efferent activity in healthy human volunteers. The studies focused particularly on the stimulation of gastric receptors using either different solutions at 37°C or 6°C including, Ispaghula husk solution, isotonic saline solution, water, or coated peppermint capsules followed by various recording intervals during the post-drink period, which provided an insight into the functional expression pattern of TRP channels on the receptive nerve endings fibres. Studies also revealed an important role of these TRP receptors in mediating different cardiovascular effects in response to the stimulation of various gastric receptors, including gastric stretch receptors, gastric receptors responding to cold, and gastric receptors activated by water hypo-osmolality and menthol. Important data have emerged regarding the effects of water drinking and isotonic saline ingestion at various temperatures on the cardiovascular efferent activities. These results may explain the differences between cardiovascular responses to hypo-osmolality combined to cold stimuli obtained with cold water drinking and cardiovascular responses to cold stimulus only after cold saline ingestion.

9. 2. Summary of Findings

In longitudinal studies where the same individuals were recorded over a time and during different sessions, the assessment of the time dependent effects on and reproducibility of HRV parameters and QTc interval when quantifying cardiac autonomic activities showed no significant changes between groups and within groups ($p>0.05$, ANOVA). Each measure at all time points recorded during the post drink period did not show significant differences from respective baseline values, and no significant variations between equal time points first versus second visits ($p>0.05$, paired t-test) were observed in both HRV parameters and QTc interval over the time course. These results indicate reproducibility of data, and no habituation effects when quantifying cardiac autonomic activities in our young healthy subjects. Therefore, autonomic modulating factors such as noise and stress were well controlled. These results were in line with observations from other studies where frequency and time domain analyses were found to be reproducible under stable conditions and during a short term analysis (van Hoogenhuyze *et al.*, 1991; Pitzalis *et al.*, 1996). Therefore, different cardiac autonomic efferent activity variations found in this thesis were associated with the effects of the stimulus, rather than being related to the time spent during the recording period or to the second exposure to the protocol procedures. This work showed a short-lived increase in sympathetic activity with a corresponding slight increase in SBP after gastric distension with 300 mL of Fybogel at body temperature, whereas gastric distension combined to cold stimulus (cold Fybogel) induced a cold mediated inhibition of sympathetic tone, probably due to an activation of TRPM8

thermoreceptors which resulted in a reduced time to onset in a decrease in HR with a corresponding decreased SBP. However, when TRPM8 thermoreceptors were activated by menthol, the decreased HR appeared after 25 minutes, maybe due to the time taken by coated capsules of peppermint oil to release its menthol content (Johnson *et al*., 2009). In addition, subjects responded to drinking 300 mL of isothermic water with unchanged HR and slight increase in BP, probably due to the hypo-osmolality mediated increased cardiac vagal activity which may have counteracted the concomitant sympathetic stimulation, suppressing the pressor effect observed in autonomic failure patients (Thayer and Lane, 2007) who are unable to elicit a buffering reflex due baroreflex impairment (Cariga *et al*., 2001; May and Jordan, 2011). This increased sympathovagal activation was not found following ingestion of the same quantity of saline solution (0.9% w/v) ingested at body temperature, which was expected to generate a greater plasma volume than water, indicating that the cardiovascular responses to water drinking were mediated by water hypo-osmolality, rather being associated with vascular volume loading effects. Conversely, the ingestion of 300 mL of cold water induced a 40 minute decrease in HR due to cumulative cold and hypo-osmolality effects which enhanced cardiac vagal tone and inhibited the hypo-osmolality mediated sympathetic tone. However, the drinking of 300 mL cold isotonic saline solution generated a 30 minute increase in cardiac vagal tone, with the corresponding 30 minute decrease in HR due to the cold effect only (without hypo-osmolality effect). The cold mediated sympathetic inhibition with both water and saline solution appeared to be reintroduced after the intra-abdominal body

temperature warmed up cold water and cold saline solution to the body temperature level.

9. 3. Final Discussion

The research presented here has led to the discovery of sympathetic activation after both gastric stretch and water drinking at body temperature which lasted 20 minutes and 40 minutes respectively, but not with isothermic saline ingestion in young healthy subjects. This sympathetic activity was inhibited by cold mediated vagal tone when the stimulation of gastric distension was associated with gastric cooling or after cold water drinking. The cold mediated vagal stimulation counteracted the sympathetic activity probably at the level of the NTS where the two branches meet for the first time during their central pathway (Kubin *et al*., 2006; Thayer and Lane, 2009). In these subjects, the slight increase in SBP in response to gastric distension, appears to be proportional to the volume of distension (Seth *et al*., 2008; Vanis *et al*., 2012) and/or to be related to good compliant arteries in our young subjects, which allowed greater decrease in splanchnic arterial resistance and an increase in superior mesenteric artery blood flow (Monahan *et al*. 2001). In response to cold water ingestion, SBP showed a classic biphasic course due to stomach cooling which tended to decrease slightly the BP during the first 15 minutes, whereas isothermic water ingestion resulted in a gradual increase during at least the first hour post-ingestion although the first 5 minutes trend were associated with the oropharyngeal sympathetic activation. The inhibitory baroreflex mechanism expected to counteract the sympathetic outflow and

causing a decrease in HR (Fadel and Raven, 2011) may have been overridden by the sympathetic activation mediated by $ß_1$- adrenergic receptors (Duncker and Bache, 2008; Stavrakis *et al.*, 2011), leading to an unchanged HR. The slight increase in SBP may be interpreted as an effect which counteracts the increased superior mesenteric artery blood flow in response to gastric distension (Seth *et al.*, 2008). The stretch induced sympathetic activation may be regarded as a direct, fast-acting neural response mediated by TRPV4, TRPP, TRPA1, and TRPV1 stretch- sensitive ion channels (Seth *et al.*, 2008; Huang, 2004; Nilius and Owsianik, 2011) which send, via vagal fibres, afferent inputs to the NTS (Pozo *et al.*, 1985; Fujimura *et al.*, 1997; Ozaki *et al.*, 1999). The efferent limb involves the sympathetic pathway which operates via splanchnic nerve fibres (Molinary *et al.*, 2003). The short-lived sympathetic activity may be related to the cessation of gastric stretch corresponding with the time Fybogel solution started to enter the duodenum as it is known to help to maintain normal digestive flow (Saha, 2014). However, when gastric distension was associated with cold temperature, the cold mediated increased cardiac vagal tone inhibited the stretch mediated sympathetic activation leading to a reduced HR, maybe with a corresponding decreased cardiac output (Scott *et al.*, 2001; Fajardo *et al.*, 2008). The cold mediated sympathetic inhibition was followed by its reintroduction within 15 minutes probably due to warming of the cold Fybogel by intra-abdominal body temperature, thereby eliminating the cold receptor mediated response, explaining the short-lived decrease in HR for about 15 minutes, between 5 and 20 minutes, the first 5 minutes being influenced by the sympathetic activation during the swallowing period. The presence of

afferent sensory neurons in isolated nodose ganglion in mouse (Fajardo *et al*., 2008) and mammalian cell lines (Story *et al*., 2003) sensitive to cold and menthol as well as the presence of a dense vagal innervation in human stomach (Zhan *et al*., 2004) despite the presence of few sensory neurons containing peptides (Berthoud *et al*., 2000), indicate that afferent vagal pathway may be the route used by TRPM8 menthol and cold sensitive receptors to convey to the NTS temperature-related inputs (Girona *et al*., 2014). In return, the NTS outputs cause the NA to send efferent stimuli to the SA node to decrease HR (Paton *et al*., 2005; Bailey *et al*., 2006). However, the concomitant sympathovagal activation after isothermic water ingestion, are reported to be mediated by TRPV4 receptors sensitive to water hypo-osmolality (Brown *et al*., 2005), which deliver through sensory vagal nerve fibres osmotic-related information to the brainstem (Fajardo *et al*, 2008) or via a spinal reflex pathway (McHugh *et al*., 2010) to elicit cardiovascular responses. Bilateral subdiaphragmatic vagotomised mice and patients with cervical cord injuries still showed a pressor response to water ingestion (McHugh *et al*., 2010), indicating a spinal transmission. Sympathetic neurotransmission is considered to be the efferent limb mediating the pressor effect associated with water ingestion (McHugh *et al*., 2010; May and Jordan, 2011). The sympathetically mediated increase in SBP with water drinking (May and Jordan, 2011) or probably after gastric stretch (Vanis *et al*., 2012) is likely to play a protective role in the maintenance of standing postprandial BP in patients with severe autonomic failure (Van Orshoven *et al*., 2004; Gentilcore, 2008). The slight increase in SBP observed in this experiment after water ingestion at body temperature, may be

a mechanism which decreases the risk for blood donation-related vasovagal reactions (VVR) which is serious complication of blood donation characterised by a rapid drop in HR and BP, resulting in decreased blood flow to the brain and fainting, such as occurs in first-time donors of blood, females, and tachycardic people (Ando *et al*., 2009). Results from other observations showed that 300 mL of water ingested 15 minutes before blood collection was associated with a decrease in risk VVR (Ando *et al*., 2009). Nevertheless, direct infusion of water into the portal vein of TRPV4$^{-/-}$ mice eliciting a robust response similar to wild animals, indicates the presence of additional osmosensitive pressor mechanisms in the portal region which may be independent of TRPV4 receptors (McHugh *et al*., 2010). However, the same amount (300 mL) of water drinking at cold temperature (6°C), showed an increase in cardiac vagal tone with a corresponding decrease in HR for about 40 minutes, consistent with a recent study (Girona *et al*., 2014) where tap water ingested at 3°C also induced an increase in vagal tone, and fall in HR for about 30-45 minutes. The transduction mechanisms of cold sensitivity in vagal afferents in mammalian cell lines (Story *et al*., 2003), indicate that the temperature of the water influences cardiac vagal activity via the activation of TRPM8 receptors, known to send temperature-related information the NTS (Girona *et al*., 2014). In addition, TRPV4 receptors known to transmit information to the brainstem on osmolality are also reported to convey temperature-related information operating between 25 and 34°C to the brainstem (Watanabe *et al*., 2002; Guler *et al*., 2002). These TRPV4 osmoreceptors may have been stimulated after cold water has been warmed up within a short time to intra-abdominal environmental

temperature levels (Girona *et al.*, 2014). The trend towards decreased SBP during the first 15 minutes due to cold mediated sympathetic inhibition, followed by a trend towards increased SBP afterward, may be due the sympathetic reintroduction and/or to peripheral skin vasoconstriction in response to cold water ingestion which is accompanied by an increase in TPR (Girona *et al.*, 2014). However, the trend towards increased SBP with isothermic water ingestion is reported to being induced through changes in CO than in TPR, indicating different autonomic mechanisms being involved as ingestion of water at body temperature results in a higher myocardial oxygen demand compared with cold water (Girona *et al.*, 2014). Around 40 minutes after cold water ingestion, the cardiovascular parameters returned near the resting level probably after the cold water has been warmed up by intra-abdominal body temperature, reducing the increased TPR. In general, different cardiovascular autonomic efferent activity variations associated with the ingestion of different stimuli are mediated by the stimulation of several TRP channels, including in TRPP, TRPV1, TRPA1, and TRPV4 receptors related to increased sympathetic nerve activity due to gastric stretch. However, the cold mediated increased vagal tone inhibits the stretch mediated sympathetic activity via TRPM8 thermoreceptors activation which also responds to menthol (McCoy et al., 2011). Finally, the cardiovascular responses to water ingestion are reported to be mediated via TRPV4 hypo-osmotic sensitive receptors (McHuhg *et al.*, 2010, Brown *et al.*, 2005).

9. 3. Final Conclusions

Studies in young healthy subjects with intact baroreflex function led to the discovery of significant cardiovascular effects after cold water ingestion, including reduced HR and a slight drop in BP which may decrease the workload to the heart**,** and could be pertinent in clinical settings where water drinks are given to cardiovascular patients, although further investigations addressing the role of water temperature are needed in human pathologies. In these subjects, 300 mL of cold water ingestion elicited sustained reduction of HR for about 40 minutes. Both cold the temperature and water hypo-osmolality are known to enhance cardiac vagal tone (MCHugh *et al*., 2010, May and Jordan, 2011). The cold temperature may have inhibited the hypo-osmolality mediated sympathetic activation, reported been activated by water ingestion, concomitantly with cardiac parasympathetic nervous system (Brown *et al*., 2005). The cold effect acts through TRPM8 receptors, while the hypo-osmolality operates via TRPV4 osmoreceptors which is also reported to convey thermosensitivity in the range between 25 to 34°C to the NTS (Watanabe *et al*., 2002), after cold water has been warmed up to intra-abdominal temperature levels (Girona *et al*., 2014), giving a combined effect of HR reduction with cold water ingestion. The potential peripheral skin vasoconstriction due to cold temperature may be a process that prevents heat loss and plays a role in warming up of cold water to intra-abdominal temperature levels, as cold water has been argued to increase thermogenesis (Girona *et al*., 2014). However, the same volume of cold saline ingestion did not have any effect on cardiac sympathetic activity, but enhanced the cardiac vagal tone with a corresponding reduction in HR for about 30

minutes. The difference found in the time course between 40 minutes HR decrease in response to cold water ingestion and 30 minutes decrease after cold saline drinking may be due to combined effects of cold and hypo-osmolality mediated increased vagal tone with cold water, whereas the HR reduction with cold saline was associated with cold effect only. Therefore, these findings provide evidence that, beside the hypo-osmolality influence in cardiovascular activities reported in this study and others (Brown *et al.*, 2005; McHugh *et al.*, 2010; May and Jordan, 2011), the temperature of water gives a combined effect on cardiac vagal tone. The increased cardiac vagal tone and decrease in HR with cold saline may also have a therapeutic benefit in clinical scenarios if the aim is to decrease the HR without activating the sympathetic nerve activity, although the time taken for the cardiovascular parameters to return near the baseline levels is shorter (30 min) than the time observed with cold water ingestion (40 min). In addition, the stimulation of TRPM8 receptors by menthol also showed a decreased HR after 25 minutes, probably due the time taken for coated capsules of peppermint oil to release its menthol content in the stomach. The antispasmodic effect of coated capsules peppermint oil is a relevant information in clinical use when treating patients with irritant bowl syndrome and gastric ulcer (Johnson *et al.*, 2009), but this finding has to be taken with caution as the ingestion of coated capsules of PO at toxic dose, estimated to 1 gram per kilogram of body weight (Baibars *et al.*, 2012), is reported to induce a dramatic decrease in BP (Nath *et al.*, 2012) due to reduction in the arterial smooth muscle tonicity (Meamarbashi, 2014).

Although healthy individuals with normal baroreflex function do not show large fluctuations in BP, water ingestion at body temperature nonetheless caused an increase in both the sympathetic and the parasympathetic nerve activity. The cardiac vagal activation may have counteracted the concomitant sympathetic stimulation, resulting in unchanged HR. These findings are compatible with the existence of osmoreceptive nerve fibres in the gut or portal circulation that can influence cardiovascular autonomic regulation in humans. Hypotonicity, and not luminal stretch, was found to be the stimulus eliciting the effects of water, as the same volume of saline ingestion failed to produce the same cardiovascular changes observed with water at body temperature, demonstrating no vascular volume loading effects.

Blockade of α1-adrenergic receptors with prazosin has been shown to attenuate the pressor response, and mice lacking NE (Dbh-/-) could not produce the response (McHugh *et al.*, 2010), indicating that the cardiovascular effects of water ingestion only occur if sympathetic efferent nerve fibres are intact. Afferent mechanisms responsible for sensing osmotic changes after water ingestion also appear to travel via spinal nerves as part of a spinal reflex mechanism, as both bilateral subdiaphragmatically vagotomized mice as well as patients with high cervical spinal cord injuries expressed a pressor response to water (McHugh *et al.*, 2010). The increase in cardiac vagal activity during this experiment appeared immediately, suggesting that TRPV4 receptors might be sensing osmolality changes in the GI tract and in the portal system despite the small volume of water ingested. This is in agreement with another observation where infusion of water into the portal vein generated cardiovascular changes,

indicating that TRPV4 might be sensing osmolality changes at the earliest junctions between GI lumen and portal circulation: mesenteric venules (McHugh *et al.*, 2010). These cardiovascular responses to water drinking may give a therapeutic benefit in the relief of different pathologies, including postprandial hypotension due to increased sympathetic enhancement and inadequate sympathetic nervous stimulation (Girona *et al.*, 2014). An American Red Cross study reported that water drinking could prevent the risk of blood donation-related vasovagal reactions (Newman *et al.*, 2007). In contrast to water ingestion, drinking of the same volume of saline solution at the body temperature did not change heart rate, cardiac vagal activity, and cardiac sympathetic tone, indicating that NaCl concentration did not have any effect on the cardiovascular activity.

However, stretching of the stomach wall by means of Fybogel in this study may have resulted in the activation of stretch receptors which induced an increase in sympathetic nerve activity and BP. Gastric stretch can be considered as a mechanism to prevent a fall in systemic blood pressure during and after the meal due to increased blood flow in the superior mesenteric artery which provides adequate oxygen to the GI tract and support an effective absorption of nutrients (Seth *et al.*, 2008). It has been suggested that the sympathetic response may increase myocardial oxygen demand sufficiently to provoke the symptoms of angina, including chest pain, probably due to an increase in the microvascular resistance directly by vasomotor nerve impulses transmitted in the vagus nerve (Mellow *et al.*, 1983; Manisty *et al.*, 2009). Therefore, the degree of gastric distension needs to be considered to avoid the occurrence of

the cardiovascular side effects, including the angina pectoris. Beyond the published gastric stretch mediated increased sympathetic activity and BP (Van Orshoven *et al.*, 2004; Vanis *et al.*, 2012), gastric distension combined with cold stimulus observed in the current study, induced a reduction in both HR and BP, probably due to cold mediated increased cardiac vagal tone and cold mediated sympathetic withdrawal. The sympathetic inhibition and the corresponding reduced HR, with a substantial decreased workload to the heart may be a relevant observation in clinical situations when patients with cardiovascular pathologies distend the stomach with cold food, although further investigations are needed to fully investigate this phenomenon.

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